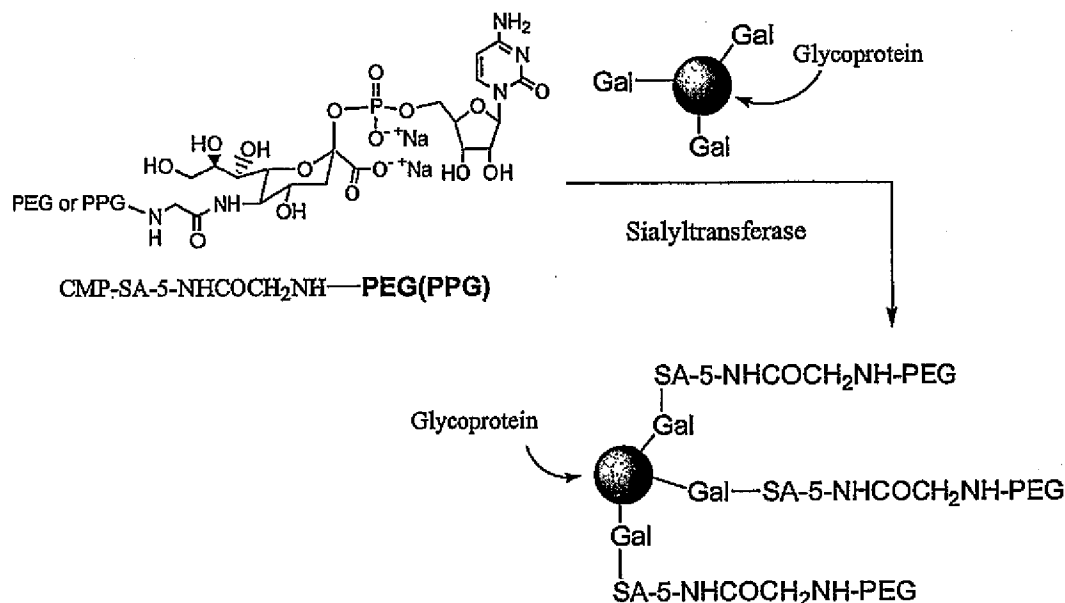
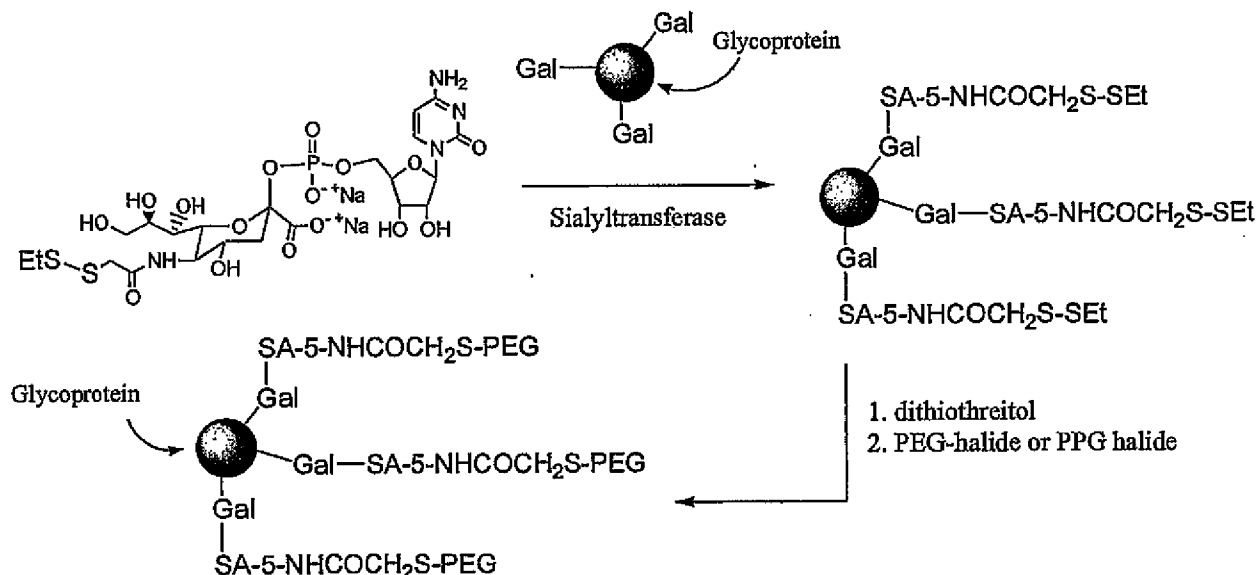


Scheme 12



In yet a further approach, summarized in Scheme 13, a masked reactive functionality is present on the sialic acid. The masked reactive group is preferably unaffected by the conditions used to attach the modified sialic acid to the peptide. After the covalent attachment of the modified sialic acid to the peptide, the mask is removed and the peptide is conjugated with an agent such as PEG, PPG, a therapeutic moiety, biomolecule or other agent. The agent is conjugated to the peptide in a specific manner by its reaction with the unmasked reactive group on the modified sugar residue.

### Scheme 13

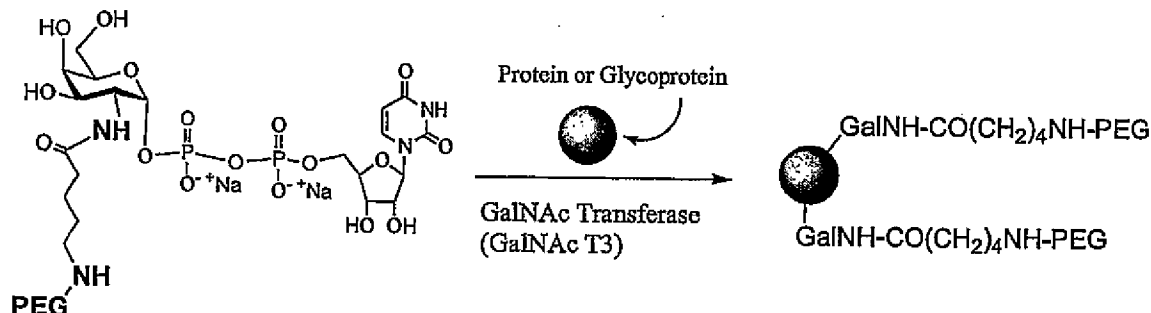


Any modified sugar can be used with its appropriate glycosyltransferase, depending on the terminal sugars of the oligosaccharide side chains of the glycopeptide (Table 3). As discussed above, the terminal sugar of the glycopeptide required for introduction of the PEGylated or PPGylated structure can be introduced naturally during expression or it can be produced post expression using the appropriate glycosidase(s), glycosyltransferase(s) or mix of glycosidase(s) and glycosyltransferase(s).

In another exemplary embodiment, a GlcNAc transferase, such as GnTI-V, is utilized to transfer PEGylated-GlcNAc to a mannose residue on a glycopeptide. In a still further exemplary embodiment, the N- and/or O-linked glycan structures are enzymatically removed from a glycopeptide to expose an amino acid or a terminal glycosyl residue that is subsequently conjugated with the modified sugar. For example, an endoglycanase is used to remove the N-linked structures of a glycopeptide to expose a terminal GlcNAc as a GlcNAc-linked-Asn on the glycopeptide. UDP-Gal-PEG and the appropriate galactosyltransferase is used to introduce the PEG- or PPG-galactose functionality onto the exposed GlcNAc.

In an alternative embodiment, the modified sugar is added directly to the peptide backbone using a glycosyltransferase known to transfer sugar residues to the peptide backbone. This exemplary embodiment is set forth in Scheme 14. Exemplary glycosyltransferases useful in practicing the present invention include, but are not limited to, GalNAc transferases (GalNAc T1-14), GlcNAc transferases, fucosyltransferases, glucosyltransferases, xylosyltransferases, mannosyltransferases and the like. Use of this approach allows the direct addition of modified sugars onto peptides that lack any carbohydrates or, alternatively, onto existing glycopeptides. In both cases, the addition of the modified sugar occurs at specific positions on the peptide backbone as defined by the substrate specificity of the glycosyltransferase and not in a random manner as occurs during modification of a protein's peptide backbone using chemical methods. An array of agents can be introduced into proteins or glycopeptides that lack the glycosyltransferase substrate peptide sequence by engineering the appropriate amino acid sequence into the peptide chain.

Scheme 14



In each of the exemplary embodiments set forth above, one or more additional chemical or enzymatic modification steps can be utilized following the conjugation of the

Table 4: Lysosomal storage diseases and associated enzymatic defects

Disease	Enzymatic Defect
Pompe disease	acid $\alpha$ -glucosidase (acid maltase)
MPSI* (Hurler disease)	$\alpha$ -L-iduronidase
MPSII (Hunter disease)	iduronate sulfatase
MPSIII (Sanfilippo)	heparan N-sulfatase
MPS IV (Morquio A)	galactose-6-sulfatase
MPS IV (Morquio B)	acid $\beta$ -galactosidase
MPS VII (Sly disease)	$\beta$ -glucuronidase
I-cell disease	N-acetylglucosamine-1-phosphotransferase
Schindler disease	$\alpha$ -N-acetylgalactosaminidase ( $\alpha$ -galactosidase B)
Wolman disease	acid lipase
Cholesterol ester storage disease	acid lipase
Farber disease	lysosomal acid ceramidase
Niemann-Pick disease	acid sphingomyelinase
Gaucher disease	glucocerebrosidase
Krabbe disease	galactosylceramidase
Fabry disease	$\alpha$ -galactosidase A
GM1 gangliosidosis	acid $\beta$ -galactosidase
Galactosialidosis	$\beta$ -galactosidase and neuraminidase
Tay-Sach's disease	hexosaminidase A
Sandhoff disease	hexosaminidase A and B

\*MPS = mucopolysaccharidosis

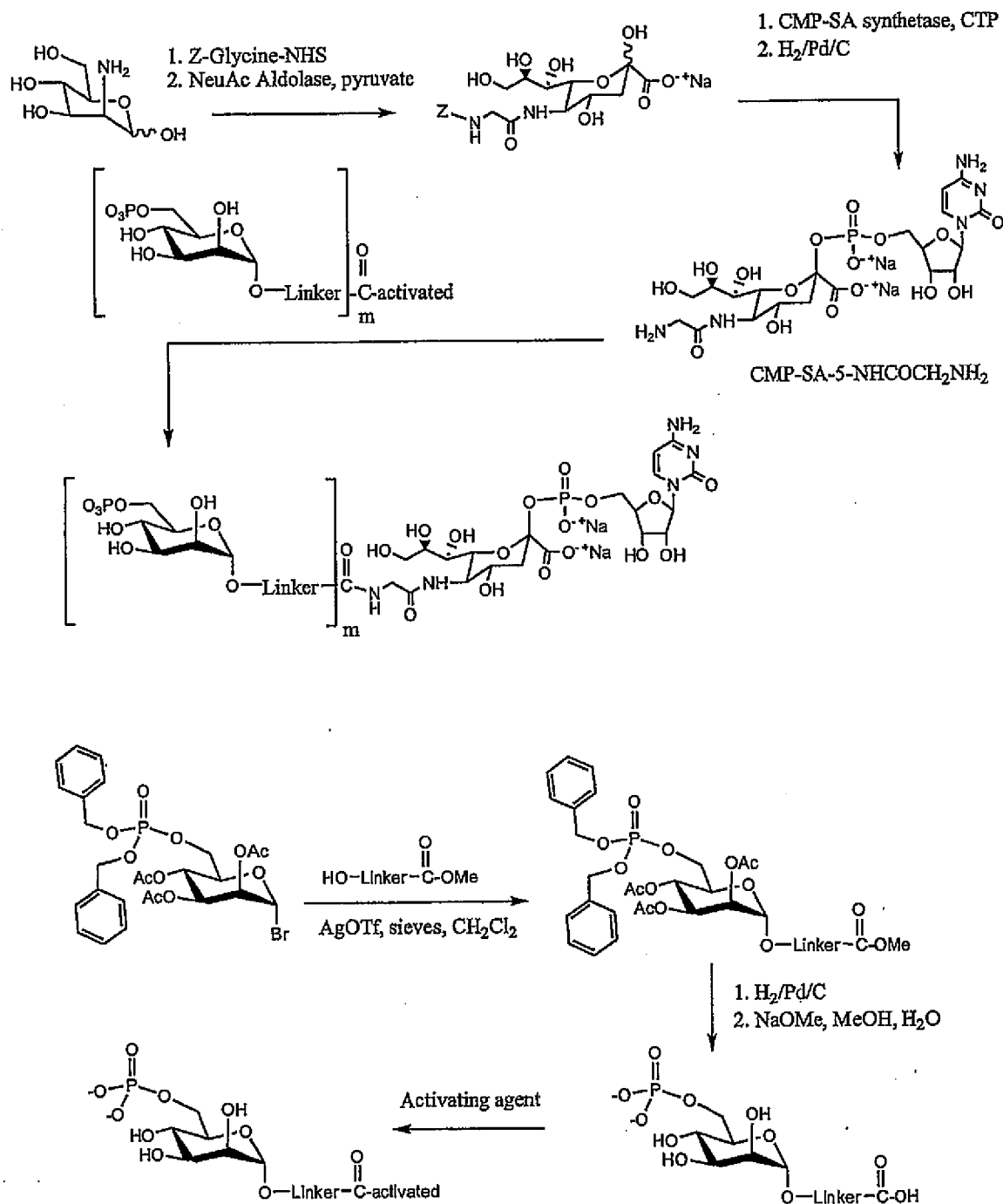
De Duve first suggested that replacement of the missing lysosomal enzyme with exogenous biologically active enzyme might be a viable approach to treatment of lysosomal storage diseases (De Duve, *Fed. Proc.* 23: 1045 (1964). Since that time, various studies have suggested that enzyme replacement therapy may be beneficial for treating various lysosomal storage diseases. The best success has been shown with individuals with type I Gaucher disease, who have been treated with exogenous enzyme ( $\beta$ -glucocerebrosidase), prepared from placenta (Ceredase™) or, more recently, recombinantly (Cerezyme™). It has been suggested that enzyme replacement may also be beneficial for treating Fabry's disease, as well as other lysosomal storage diseases. See, for example, Dawson *et al.*, *Ped. Res.* 7(8): 684-690 (1973) (*in vitro*) and Mapes *et al.*, *Science* 169: 987 (1970) (*in vivo*). Clinical trials of enzyme replacement therapy have been reported for Fabry patients using infusions of normal plasma (Mapes *et al.*, *Science* 169: 987-989 (1970)),  $\alpha$ -galactosidase A purified from placenta (Brady *et al.*, *N. Eng. J. Med.* 279: 1163 (1973)); or  $\alpha$ -galactosidase A purified from spleen or plasma (Desnick *et al.*, *Proc. Natl. Acad. Sci., USA* 76: 5326-5330 (1979)) and have

In addition to the mannose-6-phosphate, the peptides of the invention may be further derivatized with a moiety such as a water-soluble polymer, a therapeutic moiety, or an additional targeting moiety. Methods for attaching these and other groups are set forth herein. In an exemplary embodiment, the group other than mannose-6-phosphate is attached to the peptide via a derivatized sialic acid derivative according to Table 2, in which one or more of the "R" moieties is a group other than mannose-6-phosphate.

In an exemplary embodiment, a sialic acid moiety modified with a Cbz-protected glycine-based linker arm is prepared. The corresponding nucleotide sugar is prepared and the Cbz group is removed by catalytic hydrogenation. The resulting nucleotide sugar has an available, reactive amine that is contacted with an activated mannose-6-phosphate derivative, providing a mannose-6-phosphate derivatized nucleotide sugar that is useful in practicing the methods of the invention.

As shown in the scheme below (scheme 15), an exemplary activated mannose-6-phosphate derivative is formed by converting a 2-bromo-benzyl-protected phosphotriester into the corresponding triflate, *in situ*, and reacting the triflate with a linker having a reactive oxygen-containing moiety, forming an ether linkage between the sugar and the linker. The benzyl protecting groups are removed by catalytic hydrogenation, and the methyl ester of the linker is hydrolyzed, providing the corresponding carboxylic acid. The carboxylic acid is activated by any method known in the art. An exemplary activation procedure relies upon the conversion of the carboxylic acid to the N-hydroxysuccinimide ester.

Scheme 15



- In another exemplary embodiment, as shown in the scheme below (scheme 16), a N-acetylated sialic acid is converted to an amine by manipulation of the pyruvyl moiety. Thus, the primary hydroxyl is converted to a sulfonate ester and reacted with sodium azide. The azide is catalytically reduced to the corresponding amine. The sugar is

moiety includes at least one bond that is degraded *in vivo*, releasing the therapeutic peptide from the targeting agent, following delivery of the conjugate to the targeted tissue or region of the body.

5 In yet another exemplary embodiment, the *in vivo* distribution of the therapeutic moiety is altered via altering a glycoform on the therapeutic moiety without conjugating the therapeutic peptide to a targeting moiety. For example, the therapeutic peptide can be shunted away from uptake by the reticuloendothelial system by capping a terminal galactose moiety of a glycosyl group with sialic acid (or a derivative thereof) (Figures 23 and 26). Sialylation to cover terminal Gal avoids uptake of the peptide by hepatic asialoglycoprotein  
10 (ASGP) receptors, and may extend the half life of the peptide as compared with peptides having only complex glycan chains, in the absence of sialylation.

## II. Peptide/Glycopeptides of the Invention

In one embodiment, the present invention provides a composition comprising multiple  
15 copies of a single peptide having an elemental trimannosyl core as the primary glycan structure attached thereto. In preferred embodiments, the peptide may be a therapeutic molecule. The natural form of the peptide may comprise complex N-linked glycans or may be a high mannose glycan. The peptide may be a mammalian peptide, and is preferably a human peptide. In some embodiments the peptide is selected from the group consisting of an  
20 immunoglobulin, erythropoietin, tissue-type activator peptide, and others (See Figure 1).

Exemplary peptides whose glycans can be remodeled using the methods of the invention are set forth in Figure 1.

sugar (*e.g.*, N-acetylgalactosamine, galactose, mannose, GlcNAc, glucose, fucose or xylose) to a hydroxy side chain of a hydroxyamino acid, preferably serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Several exemplary embodiments of the invention are discussed below. While several of these embodiments use peptides having names carried by trademarks, and other specific peptides as the exemplary peptide, these examples are not confined to any specific peptide. The following exemplary embodiments are contemplated to include all peptide equivalents and variants of any peptide. Such variants include, but are not limited to, adding and deleting N-linked and O-linked glycosylation sites, and fusion proteins with added glycosylation sites. One of skill in the art will appreciate that the following embodiments and the basic methods disclosed therein can be applied to many peptides with equal success.

In one exemplary embodiment, the present invention provides methods for modifying Granulocyte Colony Stimulating Factor (G-CSF). Figures 27A to 27G set forth some examples of how this is accomplished using the methodology disclosed herein. In Figure 27B, a G-CSF peptide that is expressed in a mammalian cell system is trimmed back using a sialidase. The residues thus exposed are modified by the addition of a sialic acid-poly(ethylene glycol) moiety (PEG moiety), using an appropriate donor therefor and ST3Gal1. Figure 27C sets forth an exemplary scheme for modifying a G-CSF peptide that is expressed in an insect cell. The peptide is modified by adding a galactose moiety using an appropriate donor thereof and a galactosyltransferase. The galactose residues are functionalized with PEG via a sialic acid-PEG derivative, through the action of ST3Gal1. In Figure 27D, bacterially expressed G-CSF is contacted with an N-acetylgalactosamine donor and N-acetylgalactosamine transferase. The peptide is functionalized with PEG, using a PEGylated sialic acid donor and a sialyltransferase. In Figure 27E, mammalian cell expressed G-CSF is contacted with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue on the glycan on the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine-PEG. In Figure 27F, bacterially expressed G-CSF is remodeled by contacting the peptide with an endo-GalNAc enzyme under conditions where it functions in a synthetic, rather than a hydrolytic manner, thereby adding a PEG-Gal-GalNAc molecule from an activated derivative thereof. Figure 27G provides another route for remodeling bacterially expressed



molecule is PEGylated using trans-sialidase and PEGylated sialic acid-lactose complex. In Figure 28L, IFN $\alpha$ 14C expressed in a mammalian system is sialylated using a donor of sialic acid and  $\alpha$ 2,8-sialyltransferase. In Figure 28M, IFN $\alpha$ 14C expressed in insect or fungal cells first has N-acetylglucosamine added using an appropriate donor and GnT I and/or II. The molecule is then contacted with a galactosyltransferase and a galactose donor that is derivatized with a reactive sialic acid via a linker, so that the polypeptide is attached to the reactive sialic acid via the linker and the galactose residue. The polypeptide is then contacted with ST3Gal3 and transferrin, and thus becomes connected with transferrin via the sialic acid residue. In Figure 28N, IFN $\alpha$ 14C expressed in either insect or fungal cells is first treated with endoglycanase to trim back the glycosyl groups, and is then contacted with a galactosyltransferase and a galactose donor that is derivatized with a reactive sialic acid via a linker, so that the polypeptide is attached to the reactive sialic acid via the linker and the galactose residue. The molecule is then contacted with ST3Gal3 and transferrin, and thus becomes connected with transferrin via the sialic acid residue.

In another exemplary embodiment, the invention provides methods for modifying Interferon  $\alpha$ -2a or 2b (IFN $\alpha$ ), as shown in Figures 28O to 28EE. In Figure 28P, IFN $\alpha$  produced in mammalian cells is first treated with sialidase to trim back the glycosyl units, and is then PEGylated using ST3Gal3 and a PEGylated sialic acid donor. In Figure 28Q, IFN $\alpha$  expressed in insect cells is first galactosylated using an appropriate donor and a galactosyltransferase, and is then PEGylated using ST3Gal1 and a PEGylated sialic acid donor. Figure 28R offers another method for remodeling IFN $\alpha$  expressed in bacteria: PEGylated N-acetylgalactosamine is added to the protein using an appropriate donor and N-acetylgalactosamine transferase. In Figure 28S, IFN $\alpha$  expressed in mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine-PEG. In Figure 28T, IFN $\alpha$  expressed in bacteria is PEGylated using a modified enzyme Endo-N-acetylgalactosamidase, which functions in a synthetic instead of a hydrolytic manner, and using a N-acetylgalactosamine donor derivatized with a PEG moiety. In Figure 28U, N-acetylgalactosamine is first added IFN $\alpha$  using an appropriate donor and N-acetylgalactosamine transferase, and then is PEGylated using a sialyltransferase and a

29C is a scheme for modifying IFN- $\beta$  produced by insect cells. First, N-acetylglucosamine is added to IFN- $\beta$  using an appropriate donor and GnT-I and/or -II. The protein is then galactosylated using a galactose donor and a galactosyltransferase. Finally, IFN- $\beta$  is PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 29D, IFN- $\beta$  expressed in yeast is first treated with Endo-H to trim back its glycosyl chains, and is then galactosylated using a galactose donor and a galactosyltransferase, and is then PEGylated using ST3Gal3 and a donor of PEGylated sialic acid. In Figure 29E, IFN- $\beta$  produced by mammalian cells is modified by PEGylation using ST3Gal3 and a donor of sialic acid already derivatized with a PEG moiety. In Figure 29F, IFN- $\beta$  expressed in insect cells first has N-acetylglucosamine added by one or more of GnT I, II, IV, and V using a N-acetylglucosamine donor, and then is galactosylated using a galactose donor and a galactosyltransferase, and is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 29G, IFN- $\beta$  expressed in yeast is first treated with mannosidases to trim back the mannosyl units, then has N-acetylglucosamine added using a N-acetylglucosamine donor and one or more of GnT I, II, IV, and V. The protein is further galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a PEG-sialic acid donor. In Figure 29H, mammalian cell expressed IFN- $\beta$  is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 29I, IFN- $\beta$  expressed in a mammalian system is PEGylated using a donor of PEG-sialic acid and  $\alpha$ 2,8-sialyltransferase. In Figure 29J, IFN- $\beta$  expressed by mammalian cells is first treated with sialidase to trim back its terminal sialic acid residues, and then PEGylated using trans-sialidase and a donor of PEGylated sialic acid. In Figure 29K, IFN- $\beta$  expressed in mammalian cells is first treated with sialidase to trim back terminal sialic acid residues, then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and then sialylated using ST3Gal3 and a sialic acid donor. In Figure 29L, IFN- $\beta$  expressed in mammalian cells is first treated with sialidase and galactosidase to trim back the glycosyl chains, then galactosylated using a galactose donor and an  $\alpha$ -galactosyltransferase, and then PEGylated using ST3Gal3 or a sialyltransferase and a donor of PEG-sialic acid. In Figure 29M, IFN- $\beta$  expressed in mammalian cells is first treated with sialidase to trim back the glycosyl units. It is then

Factor IX produced by mammalian cells is first treated with sialidase to trim back the terminal sialic acid residues, and is then PEGylated with ST3Gal3 using a PEG-sialic acid donor. In Figure 31C, Factor IX expressed by mammalian cells is first treated with sialidase to trim back the terminal sialic acid residues, it is then PEGylated using ST3Gal3 and a PEG-sialic acid donor, and further sialylated using ST3Gal1 and a sialic acid donor. Another scheme for remodeling mammalian cell produced Factor IX can be found in Figure 31D. The polypeptide is first treated with sialidase to trim back the terminal sialic acid residues, then galactosylated using a galactose donor and a galactosyltransferase, further sialylated using a sialic acid donor and ST3Gal3, and then PEGylated using a donor of PEGylated sialic acid and ST3Gal1. In Figure 31E, Factor IX that is expressed in a mammalian system is PEGylated through the process of sialylation catalyzed by ST3Gal3 using a donor of PEG-sialic acid. In Figure 31F, Factor IX expressed in mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. Figure 31G provides an additional method of modifying Factor IX. The polypeptide, produced by mammalian cells, is PEGylated using a donor of PEG-sialic acid and  $\alpha$  2,8-sialyltransferase.

In another exemplary embodiment, the invention provides methods for modification of Follicle Stimulating Hormone (FSH). Figures 32A to 32J present some examples. In Figure 32B, FSH is expressed in a mammalian system and modified by treatment of sialidase to trim back terminal sialic acid residues, followed by PEGylation using ST3Gal3 and a donor of PEG-sialic acid. In Figure 32C, FSH expressed in mammalian cells is first treated with sialidase to trim back terminal sialic acid residues, then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and then sialylated using ST3Gal3 and a sialic acid donor. Figure 32D provides a scheme for modifying FSH expressed in a mammalian system. The polypeptide is treated with sialidase and galactosidase to trim back its sialic acid and galactose residues, then galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 32E, FSH expressed in mammalian cells is modified in the following procedure: FSH is first treated with sialidase to trim back the sialic acid residues, then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and is then sialylated using ST3Gal3 and a sialic acid donor.

and a galactose donor. The terminal galactose residues of the N-linked glycosyl units are "capped" with sialic acid, using ST3Gal3 and a sialic acid donor. The terminal galactose residues are functionalized with a sialic acid bearing a PEG moiety, using an appropriate sialic acid donor and ST3Gal1. In Figure 33E, EPO that is expressed in a mammalian cell system is remodeled by functionalizing the N-linked glycosyl residues with a PEG-derivatized sialic acid moiety. The peptide is contacted with ST3Gal3 and an appropriately modified sialic acid donor. In Figure 33F, EPO that is expressed in an insect cell system is remodeled by adding one or more terminal N-acetylglucosamine residues by contacting the peptide with a N-acetylglucosamine donor and one or more of GnTI, GnTII, and GnTV.

The peptide is then PEGylated by contacting it with a PEGylated galactose donor and a galactosyltransferase. In Figure 33G, EPO that is expressed in an insect cell system is remodeled by the addition of terminal N-acetylglucosamine residues, using an appropriate N-acetylglucosamine donor and one or more of GnTI, GnTII, and GnTV. A galactosidase that is made to operate in a synthetic, rather than a hydrolytic manner is utilized to add an activated PEGylated galactose donor to the N-acetylglucosamine residues. In Figure 33H, a mutant EPO expressed in mammalian cells is remodeled by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine-PEG. Figure 33I sets forth an exemplary remodeling pathway for a mutant EPO that is expressed in a mammalian cell system. PEG is added to the glycosyl residue using a PEG-modified sialic acid and an  $\alpha$ 2,8-sialyltransferase. Figure 33J sets forth another exemplary remodeling pathway for a mutant EPO that is expressed in a mammalian cell system. The sialic acid is added to the glycosyl residue with a sialic acid donor and an  $\alpha$ 2,8-sialyltransferase.

In another exemplary embodiment, the invention provides methods for modifying granulocyte-macrophage colony-stimulating factor (GM-CSF), as shown in Figures 34A to 34K. In Figure 34B, GM-CSF expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 34C, GM-CSF expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and then is further sialylated using a sialic acid donor and ST3Gal1 and/or

galactosylated using a galactose donor and a galactosyltransferase. Then, IFN $\gamma$  is PEGylated using a donor of PEG-sialic acid and ST3Gal3. In Figure 35E, IFN $\gamma$  that is expressed in a mammalian system is first treated with sialidase to trim back terminal sialic acid residues. The polypeptide is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid, and is further sialylated with ST3Gal3 and a sialic acid donor. Figure 35F describes another method for modifying IFN $\gamma$  expressed in a mammalian system. The protein is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 35G, IFN $\gamma$  expressed in mammalian cells is remodeled by addition of sialic acid using a sialic acid donor and an  $\alpha$  2,8-sialyltransferase. In Figure 35I, IFN $\gamma$  expressed in insect or fungal cells is modified by addition of N-acetylglucosamine using an appropriate donor and one or more of GnT I, II, IV, and V. The protein is further modified by addition of PEG moieties using a donor of PEGylated galactose and a galactosyltransferase. Figure 35J offers a method for modifying IFN $\gamma$  expressed in yeast. The polypeptide is first treated with endoglycanase to trim back the saccharide chains, and then galactosylated using a galactose donor and a galactosyltransferase. Then, IFN $\gamma$  is PEGylated using a donor of PEGylated sialic acid and ST3Gal3. In Figure 35K, IFN $\gamma$  produced by mammalian cells is modified as follows: the polypeptide is first contacted with ST3Gal3 and a donor of sialic acid that is derivatized with a reactive galactose via a linker, so that the polypeptide is attached to the reactive galactose via the linker and sialic acid residue. The polypeptide is then contacted with a galactosyltransferase and transferrin pre-treated with endoglycanase, and thus becomes connected with transferrin via the galactose residue. In the scheme illustrated by Figure 35L, IFN $\gamma$ , which is expressed in a mammalian system, is modified via the action of ST3Gal3: PEGylated sialic acid is transferred from a suitable donor to IFN $\gamma$ . Figure 35M is an example of modifying IFN $\gamma$  expressed in insect or fungal cells, where PEGylation of the polypeptide is achieved by transferring PEGylated N-acetylglucosamine from a donor to IFN $\gamma$  using GnT I and/or II. In Figure 35N, IFN $\gamma$  expressed in a mammalian system is remodeled with addition of PEGylated sialic acid using a suitable donor and an  $\alpha$  2,8-sialyltransferase.

In another exemplary embodiment, the invention provides methods for modifying  $\alpha_1$  anti-trypsin ( $\alpha_1$ -protease inhibitor). Some such examples can be found in Figures 36A to

acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as one or more mannose-6-phosphate groups. In Figure 37G, Cerezyme<sup>TM</sup> expressed in mammalian cells is sialylated using a sialic acid donor and  $\alpha$  2,8-sialyltransferase. In Figure 37I, Cerezyme<sup>TM</sup> expressed in insect cells first has N-acetylglucosamine added using a suitable donor and one or more of GnT I, II, IV, and V, and then is PEGylated using a galactosyltransferase and a donor of PEG-galactose. In Figure 37J, Cerezyme<sup>TM</sup> expressed in yeast is first treated with endoglycanase to trim back the glycosyl groups, then galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 37K, Cerezyme<sup>TM</sup> expressed in mammalian cells is first contacted with ST3Gal3 and two reactive sialic acid residues connected via a linker, so that the polypeptide is attached to one reactive sialic acid via the linker and the second sialic acid residue. The polypeptide is then contacted with ST3Gal3 and desialylated transferrin, and thus becomes connected with transferrin. Then, the polypeptide is sialylated using a sialic acid donor and ST3Gal3.

In another exemplary embodiment, the invention provides methods for modifying Tissue-Type Plasminogen Activator (TPA) and its mutant. Several specific modification schemes are presented in Figures 38A to 38W. Figure 38B illustrates one modification procedure: after TPA is expressed by mammalian cells, it is treated with one or more of mannosidase(s) and sialidase to trim back mannosyl and/or sialic acid residues. Terminal N-acetylglucosamine is then added by contacting the polypeptide with a suitable donor of N-acetylglucosamine and one or more of GnT I, II, IV, and V. TPA is further galactosylated using a galactose donor and a galactosyltransferase. Then, PEG is attached to the molecule by way of sialylation catalyzed by ST3Gal3 and using a donor of sialic acid derivatized with a PEG moiety. In Figure 38C, TPA is expressed in insect or fungal cells. The modification includes the steps of addition of N-acetylglucosamine using an appropriate donor of N-acetylglucosamine and GnT I and/or II; galactosylation using a galactose donor and a galactosyltransferase; and attachment of PEG by way of sialylation using ST3Gal3 and a donor of sialic acid derivatized with PEG. In Figure 38D, TPA is expressed in yeast and subsequently treated with endoglycanase to trim back the saccharide chains. The polypeptide is further PEGylated via the action of a galactosyltransferase, which catalyzes the transfer of a PEG-galactose from a donor to TPA. In Figure 38E, TPA is expressed in insect or yeast

expressed in bacteria is modified with N-acetylgalactosamine using a proper donor and N-acetylgalactosamine transferase, followed by a step of PEGylation with a PEG-sialic acid donor and a sialyltransferase. Figure 39E offers another scheme of modifying IL-2 produced by a mammalian system. The polypeptide is modified by capping appropriate terminal  
5 residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. Figure 39F illustrates an example of remodeling IL-2 expressed by *E. coli*. The polypeptide is PEGylated using a reactive N-acetylgalactosamine complex derivatized with a PEG group and an enzyme that is  
10 modified so that it functions as a synthetic enzyme rather than a hydrolytic one. In Figure 39G, IL-2 expressed by bacteria is modified by addition of PEGylated N-acetylgalactosamine using a proper donor and N-acetylgalactosamine transferase.

In another exemplary embodiment, the invention provides methods for modifying Factor VIII, as shown in Figures 40A to 40N. In Figure 40B, Factor VIII expressed in  
15 mammalian cells is first treated with sialidase to trim back the sialic acid residues, and is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 40C, Factor VIII expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, then PEGylated using ST3Gal3 and a proper donor, and is then further sialylated using ST3Gal1 and a sialic acid donor.

In Figure 40E, mammalian cell produced Factor VIII is modified by the single step of PEGylation, using ST3Gal3 and a donor of PEGylated sialic acid. Figure 40F offers another example of modification of Factor VIII that is expressed by mammalian cells. The protein is PEGylated using ST3Gal1 and a donor of PEGylated sialic acid. In Figure 40G, mammalian cell expressed Factor VIII is remodeled following another scheme: it is PEGylated using  $\alpha$   
20 2,8-sialyltransferase and a donor of PEG-sialic acid. In Figure 40 I, Factor VIII produce by mammalian cells is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG. In Figure 40J, Factor VIII expressed by mammalian  
25 cells is first treated with Endo-H to trim back glycosyl groups. It is then PEGylated using a galactosyltransferase and a donor of PEG-galactose. In Figure 40K, Factor VIII expressed in

sialylated using a sialic acid donor and  $\alpha$  2,8-sialyltransferase. In Figure 41I, urokinase expressed in insect cells is modified in the following steps: first, N-acetylglucosamine is added to the polypeptide using a suitable donor of N-acetylglucosamine and one or more of GnT I, II, IV, and V; then PEGylated galactose is added, using a galactosyltransferase and a donor of PEG-galactose. In Figure 41J, urokinase expressed in yeast is first treated with endoglycanase to trim back glycosyl groups, then galactosylated using a galactose donor and a galactosyltransferase, and then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 41K, urokinase expressed in mammalian cells is first contacted with ST3Gal3 and two reactive sialic acid residues that are connected via a linker, so that the polypeptide is attached to one reactive sialic acid via the linker and second sialic acid residue. The polypeptide is then contacted with ST3Gal1 and desialylated urokinase produced in mammalian cells, and thus becomes connected with a second molecule of urokinase. Then, the whole molecule is further sialylated using a sialic donor and ST3Gal1 and/or ST3Gal3. In Figure 41L, isolated urokinase is first treated with sulfohydrolase to remove sulfate groups, and is then PEGylated using a sialyltransferase and a donor of PEG-sialic acid. In Figure 41M, isolated urokinase is first treated with sulfohydrolase and hexosaminidase to remove sulfate groups and hexosamine groups, and then PEGylated using a galactosyltransferase and a donor of PEG-galactose.

In another exemplary embodiment, the invention provides methods for modifying DNase I, as shown in Figures 42A to 42K. In Figure 42B, DNase I is expressed in a mammalian system and modified in the following steps: first, the protein is treated with sialidase to trim back the sialic acid residues; then the protein is PEGylated with ST3Gal3 using a donor of PEG-sialic acid. In Figure 42C, DNase I expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, then PEGylated with ST3Gal3 using a PEG-sialic acid donor, and is then sialylated using ST3Gal3 and a sialic acid donor. In Figure 42D, DNase I expressed in a mammalian system is first exposed to sialidase and galactosidase to trim back the glycosyl groups, then galactosylated using a galactose donor and an  $\alpha$ -galactosyltransferase, and then PEGylated using ST3Gal3 or sialyltransferase and a donor of PEG-sialic acid. In Figure 42E, DNase I expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues, then PEGylated using ST3Gal3 and a PEG-sialic acid donor, and then sialylated with ST3Gal3 using a sialic acid donor. In



human growth hormone-mucin fusion protein: the fusion protein is PEGylated through the action of N-acetylgalactosamine transferase using a donor of PEGylated N-acetylgalactosamine. Figure 45J provides a further remodeling scheme for human growth hormone-mucin fusion protein. The fusion protein is first contacted with N-

5 acetylgalactosamine transferase and a donor of N-acetylgalactosamine that is derivatized with a reactive sialic acid via a linker, so that the fusion protein is attached to the reactive sialic acid via the linker and N-acetylgalactosamine. The fusion protein is then contacted with a sialyltransferase and asialo-transferrin, and thus becomes connected with transferrin via the sialic acid residue. Then, the fusion protein is capped with sialic acid residues using  
10 ST3Gal3 and a sialic acid donor. In Figure 45K, yet another scheme is given for modification of human growth hormone(N) produced in bacteria. The polypeptide is first contacted with NHS-CO-linker-SA-CMP and becomes coupled with the reactive sialic acid through the linker. The polypeptide is then contacted with ST3Gal3 and asialo-transferrin and becomes linked to transferrin via the sialic acid residue. Then, the polypeptide is  
15 sialylated using ST3Gal3 and a sialic acid donor.

In another exemplary embodiment, the invention provides methods for remodeling TNF receptor IgG fusion protein (TNFR-IgG, or Enbrel™), as shown in Figures 46A to G. Figure 46B illustrates a modification procedure in which TNFR-IgG, expressed in a mammalian system is first sialylated with a sialic acid donor and a sialyltransferase,

20 ST3Gal1; the fusion protein is then galactosylated with a galactose donor and a galactosyltransferase; then, the fusion protein is PEGylated via the action of ST3Gal3 and a donor of sialic acid derivatized with PEG. In Figure 46C, TNFR-IgG expressed in mammalian cells is initially treated with sialidase to trim back sialic acid residues. PEG moieties are subsequently attached to TNFR-IgG by way of transferring PEGylated sialic  
25 acid from a donor to the fusion protein in a reaction catalyzed by ST3Gal1. In Figure 46D, TNFR-IgG is expressed in a mammalian system and modified by addition of PEG through the galactosylation process, which is mediated by a galactosyltransferase using a PEG-galactose donor. In Figure 46E, TNFR-IgG is expressed in a mammalian system. The first step in remodeling of the fusion protein is adding O-linked sialic acid residues using a sialic  
30 acid donor and a sialyltransferase, ST3Gal1. Subsequently, PEGylated galactose is added to the fusion protein using a galactosyltransferase and a suitable donor of galactose with PEG

PEGylated galactose using a suitable donor and a galactosyltransferase. In Figure 49D, Remicade™ expressed in fungi is first treated with Endo-H to trim back the glycosyl chains, then galactosylated using a galactose donor and a galactosyltransferase, and then conjugated to a radioisotope using ST3Gal3 and a reactive sialic acid derivatized with the radioisotope.

5 In another exemplary embodiment, the invention provides methods for modifying Reopro, which is mutated to contain an N glycosylation site. Figures 50A to 50L contain such examples. In Figure 50B, Reopro expressed in a mammalian system is first treated with sialidase to trim back the sialic acid residues, and the PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 50C, Reopro expressed in insect cells is modified by addition  
10 of PEGylated N-acetylglucosamine using an appropriate donor and GnT I and/or II. In Figure 50D, Reopro expressed in yeast is first treated with Endo-H to trim back the glycosyl groups. Subsequently, the protein is PEGylated using a galactosyltransferase and a donor of PEG-galactose. In Figure 50F, Reopro expressed in mammalian cells is first treated with sialidase to trim back the sialic acid residues and then PEGylated with ST3Gal1 using a  
15 donor of PEGylated sialic acid. In Figure 50G, Reopro expressed in insect cells is modified by PEGylation using a galactosyltransferase and a donor of PEG-galactose. In Figure 50H, Reopro expressed in bacterial first has N-acetylgalactosamine added using N-acetylgalactosamine transferase and a suitable donor. The protein is then PEGylated using a sialyltransferase and a donor of PEG-sialic acid. In Figure 50J, Reopro expressed in bacteria  
20 is modified in a different scheme: it is PEGylated via the action of N-acetylgalactosamine transferase, using a donor of PEGylated N-acetylgalactosamine. In Figure 50K, bacterially expressed Reopro is modified in yet another method: first, the polypeptide is contacted with N-acetylgalactosamine transferase and a donor of N-acetylgalactosamine that is derivatized with a reactive sialic acid via a linker, so that the polypeptide is attached to the reactive sialic  
25 acid via the linker and N-acetylgalactosamine. The polypeptide is then contacted with ST3Gal3 and asialo-transferrin and thus becomes connected with transferrin via the sialic acid residue. Then, the polypeptide is capped with sialic acid residues using a proper donor and ST3Gal3. Figure 50L offers an additional scheme of modifying bacterially expressed Reopro. The polypeptide is first exposed to NHS-CO-linker-SA-CMP and becomes  
30 connected with the reactive sialic acid through the linker. The polypeptide is then contacted with ST3Gal3 and asialo-transferrin and thus becomes connected with transferrin via the

invention further encompasses peptides that have N-linked glycan chains at other recognition sites where the N-linked chains are added using natural or recombinant glycosyltransferases.

Since the recognition site for N-linked glycosylation of a peptide is known, it is within the skill of persons in the art to create mutated primary peptide sequences wherein a native N-linked glycosylation recognition site is removed, or alternatively or in addition, one or more additional N-glycosylation recognition sites are created. Most simply, an asparagine residue can be removed from the primary sequence of the peptide thereby removing the attachment site for a glycan, thus removing one glycan from the mature peptide. For example, a native recognition site with the sequence of asparagine-serine-serine can be genetically engineered to have the sequence leucine-serine-serine, thus eliminating a N-linked glycosylation site at this position.

Further, an N-linked glycosylation site can be removed by altering the residues in the recognition site so that even though the asparagine residue is present, one or more of the additional recognition residues are absent. For example, a native sequence of asparagine-serine-serine can be mutated to asparagine-serine-lysine, thus eliminating an N-glycosylation site at that position. In the case of N-linked glycosylation sites comprising residues other than the typical recognition sites described above, the skilled artisan can determine the sequence and residues required for recognition by the appropriate glycosyltransferase, and then mutate at least one residue so the appropriate glycosyltransferase no longer recognizes that site. In other words, it is well within the skill of the artisan to manipulate the primary sequence of a peptide such that glycosylation sites are either created or are removed, or both, thereby generating a peptide having an altered glycosylation pattern. The invention should therefore not be construed to be limited to any primary peptide sequence provided herein as the sole sequence for glycan remodeling, but rather should be construed to include any and all peptide sequences suitable for glycan remodeling.

To create a mutant peptide, the nucleic acid sequence encoding the primary sequence of the peptide is altered so that native codons encoding native amino acid residues are mutated to generate a codon encoding another amino acid residue. Techniques for altering nucleic acid sequence are common in the art and are described for example in any well-known molecular biology manual.

selected which include recombinant vectors bearing the mutated sequence arrangement. A genetic selection scheme was devised by Kunkel et al. (1987, Kunkel et al., Methods Enzymol. 154:367-382) to enrich for clones incorporating the mutagenic oligonucleotide. Alternatively, the use of PCR<sup>TM</sup> with commercially available thermostable enzymes such as Taq polymerase may be used to incorporate a mutagenic oligonucleotide primer into an amplified DNA fragment that can then be cloned into an appropriate cloning or expression vector. The PCR<sup>TM</sup>-mediated mutagenesis procedures of Tomic et al. (1990, Nucl. Acids Res., 12:1656) and Upender et al. (1995, Biotechniques, 18:29-31) provide two examples of such protocols. A PCR<sup>TM</sup> employing a thermostable ligase in addition to a thermostable polymerase may also be used to incorporate a phosphorylated mutagenic oligonucleotide into an amplified DNA fragment that may then be cloned into an appropriate cloning or expression vector. The mutagenesis procedure described by Michael (1994, Biotechniques 16:410-412) provides an example of one such protocol.

Not all Asn-X-Ser/Thr sequences are N-glycosylated suggesting the context in which the motif is presented is important. In another approach, libraries of mutant peptides having novel N-linked consensus sites are created in order to identify novel N-linked sites that are glycosylated *in vivo* and are beneficial to the activity, stability or other characteristics of the peptide.

As noted previously, the consensus sequence for the addition of N-linked glycan chains in glycoproteins is Asn-X-Ser/Thr where X can be any amino acid. The nucleotide sequence encoding the amino acid two positions to the carboxyl terminal side of the Asn may be mutated to encode a Ser and/or Thr residue using standard procedures known to those of ordinary skill in the art. As stated above not all Asn-X-Ser/Thr sites are modified by the addition of glycans. Therefore, each recombinant mutated glycoprotein must be expressed in a fungal, yeast or animal or mammalian expression system and analyzed for the addition of an N-linked glycan chain. The techniques for the characterization of glycosylation sites are well known to one skilled in the art. Further, the biological function of the mutated recombinant glycoprotein can be determined using assays standard for the particular protein being examined. Thus, it becomes a simple matter to manipulate the primary sequence of a peptide and identify novel glycosylation sites contained therein, and further determine the effect of the novel site on the biological activity of the peptide.

In yet another approach, advantageous sites for new O-linked sites may be found in a peptide by creating libraries of the peptide containing various new O-linked sites. For example, the consensus amino acid sequence for N-acetylgalactosamine addition by an N-acetylgalactosaminyltransferase depends on the specific transferase used. The amino acid sequence of a peptide may be scanned to identify contiguous groups of amino acids that can be mutated to generate potential sites for addition of O-linked glycan chains. These mutations can be generated using standard procedures known to those of ordinary skill in the art as described previously. In order to determine if any discovered glycosylation site is actually glycosylated, each recombinant mutated peptide is then expressed in a suitable expression system and is subsequently analyzed for the addition of the site and/or the presence of an O-linked glycan chain.

#### C. Chemical synthesis of peptides

While the primary structure of peptides useful in the invention can be generated most efficiently in a cell-based expression system, it is within the scope of the present invention that the peptides may be generated synthetically. Chemical synthesis of peptides is well known in the art and include, without limitation, stepwise solid phase synthesis, and fragment condensation either in solution or on solid phase. A classic stepwise solid phase synthesis of involves covalently linking an amino acid corresponding to the carboxy-terminal amino acid of the desired peptide chain to a solid support and extending the peptide chain toward the amino end by stepwise coupling of activated amino acid derivatives having activated carboxyl groups. After completion of the assembly of the fully protected solid phase bound peptide chain, the peptide-solid phase covalent attachment is cleaved by suitable chemistry and the protecting groups are removed to yield the product peptide. See, R. Merrifield, Solid Phase Peptide Synthesis: The Synthesis of a Tetrapeptide, J. Am. Chem. Soc., 85:2149-2154 (1963). The longer the peptide chain, the more challenging it is to obtain high-purity well-defined products. Due to the production of complex mixtures, the stepwise solid phase synthesis approach has size limitations. In general, well-defined peptides of 100 contiguous amino acid residues or more are not routinely prepared via stepwise solid phase synthesis.

The segment condensation method involves preparation of several peptide segments by the solid phase stepwise method, followed by cleavage from the solid phase and

purification of these maximally protected segments. The protected segments are condensed one-by-one to the first segment, which is bound to the solid phase.

The peptides useful in the present invention may be synthesized by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. These synthesis methods are well-known to those of skill in the art (see, for example, Merrifield, J. Am. Chem. Soc. 85:2149 (1963), Stewart et al., "Solid Phase Peptide Synthesis" (2nd Edition), (Pierce Chemical Co. 1984), Bayer and Rapp, Chem. Pept. Prot. 3:3 (1986), Atherton et al., Solid Phase Peptide Synthesis: A Practical Approach (IRL Press 1989), Fields and Colowick, "Solid-Phase Peptide Synthesis," Methods in Enzymology Volume 289 (Academic Press 1997), and Lloyd-Williams et al., Chemical Approaches to the Synthesis of Peptides and Peptides (CRC Press, Inc. 1997)). Variations in total chemical synthesis strategies, such as "native chemical ligation" and "expressed peptide ligation" are also standard (see, for example, Dawson et al., Science 266:776 (1994), Hackeng et al., Proc. Nat'l Acad. Sci. USA 94:7845 (1997), Dawson, Methods Enzymol. 287: 34 (1997), Muir et al, Proc. Nat'l Acad. Sci. USA 95:6705 (1998), and Severinov and Muir, J. Biol. Chem. 273:16205 (1998)). Also useful are the solid phase peptide synthesis methods developed by Gryphon Sciences, South San Francisco, CA. See, U.S. Patent Nos. 6,326,468, 6,217,873, 6,174,530, and 6,001,364, all of which are incorporated in their entirety by reference herein.

#### 20 D. Post-translational modifications

It will be appreciated to one of ordinary skill in the art that peptides may undergo post-translational modification besides the addition of N-linked and/or O-linked glycans thereto. It is contemplated that peptides having post-translational modifications other than glycosylation can be used as peptides in the invention, as long as the desired biological activity or function of the peptide is maintained or improved. Such post-translational modifications may be natural modifications usually carried out *in vivo*, or engineered modifications of the peptide carried out *in vitro*. Contemplated known modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation,

maleimides. Derivatizing agents such as methyl-3-[9p-azidophenyl]dithiopropioimide  
yield photoactivatable intermediates that are capable of forming crosslinks in the presence of  
light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide activated  
carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287 and 3,691,016  
5 may be employed for peptide immobilization.

#### E. Fusion peptides/peptides

Peptides useful in the present invention may comprise fusion peptides. Fusion  
peptides are particularly advantageous where biological and/or functional characteristics of  
10 two peptides are desired to be combined in one peptide molecule. Such fusion peptides can  
present combinations of biological activity and function that are not found in nature to create  
novel and useful molecules of therapeutic and industrial applications. Biological activities of  
interest include, but are not limited to, enzymatic activity, receptor and/or ligand activity,  
immunogenic motifs, and structural domains.

15 Such fusion peptides are well known in the art, and the methods of creation will be  
well-known to those in the art. For example, a human  $\alpha$ -interferon—human fusion peptide  
has been made wherein the resulting peptide has the therapeutic benefits of  $\alpha$ -interferon  
combined with the long circulating life of albumin, thereby creating a therapeutic  
composition that allows reduced dosing frequency and potentially reduced side effects in  
20 patients. See, Albuferon™ from Human Genome Sciences, Inc. and U.S. Patent No.  
5,766,883. Other fusion peptides include antibody molecules that are described elsewhere  
herein.

#### F. Generation of smaller “biologically active” molecules

25 The peptides used in the invention may be variants of native peptides, wherein a  
fragment of the native peptide is used in place of the full length native peptide. In addition,  
pre-pro-, and pre-peptides are contemplated. Variant peptides may be smaller in size than the  
native peptide, and may comprise one or more domains of a larger peptide. Selection of  
specific peptide domains can be advantageous when the biological activity of certain domains  
30 in the peptide is desired, but the biological activity of other domains in the peptide is not  
desired. Also included are truncations of the peptide and internal deletions which may

enhance the desired therapeutic effect of the peptide. Any such forms of a peptide is contemplated to be useful in the present invention provided that the desired biological activity of the peptide is preserved.

Shorter versions of peptides may have unique advantages not found in the native peptide. In the case of human albumin, it has been found that a truncated form comprising as little as 63% of the native albumin peptide is advantageous as a plasma volume expander. The truncated albumin peptide is considered to be better than the native peptide for this therapeutic purpose because an individual peptide dose of only one-half to two-thirds that of natural-human serum albumin, or recombinant human serum albumin is required for the equivalent colloid osmotic effect. See U.S. Patent No. 5,380,712, the entirety of which is incorporated by reference herein.

Smaller "biologically active" peptides have also been found to have enhanced therapeutic activity as compared to the native peptide. The therapeutic potential of IL-2 is limited by various side effects dominated by the vascular leak syndrome. A shorter chemically synthesized version of the peptide consisting of residues 1-30 corresponding to the entire  $\alpha$ -helix was found to fold properly and contain the natural IL-2 biological activity with out the attending side effects.

#### G. Generation of novel peptides

The peptide of the invention may be derived from a primary sequence of a native peptide, or may be engineered using any of the many means known to those of skill in the art. Such engineered peptides can be designed and/or selected because of enhanced or novel properties as compared with the native peptide. For example, peptides may be engineered to have increased enzyme reaction rates, increased or decreased binding affinity to a substrate or ligand, increased or decreased binding affinity to a receptor, altered specificity for a substrate, ligand, receptor or other binding partner, increased or decreased stability *in vitro* and/or *in vivo*, or increased or decreased immunogenicity in an animal.

#### H. Mutations

##### 1. Rational design mutation

The peptides useful in the methods of the invention may be mutated to enhance a desired biological activity or function, to diminish an undesirable property of the peptide, and/or to add novel activities or functions to the peptide. "Rational peptide design" may be



used to generate such altered peptides. Once the amino acid sequence and structure of the peptide is known and a desired mutation planned, the mutations can be made most conveniently to the corresponding nucleic acid codon which encodes the amino acid residue that is desired to be mutated. One of skill in the art can easily determine how the nucleic acid sequence should be altered based on the universal genetic code, and knowledge of codon preferences in the expression system of choice. A mutation in a codon may be made to change the amino acid residue that will be polymerized into the peptide during translation. Alternatively, a codon may be mutated so that the corresponding encoded amino acid residue is the same, but the codon choice is better suited to the desired peptide expression system. For example, cys-residues may be replaced with other amino acids to remove disulfide bonds from the mature peptide, catalytic domains may be mutated to alter biological activity, and in general, isoforms of the peptide can be engineered. Such mutations can be point mutations, deletions, insertions and truncations, among others.

Techniques to mutate specific amino acids in a peptide are well known in the art. The technique of site-directed mutagenesis, discussed above, is well suited for the directed mutation of codons. The oligonucleotide-mediated mutagenesis method is also discussed in detail in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, starting at page 15.51). Systematic deletions, insertions and truncations can be made using linker insertion mutagenesis, digestion with nuclease Bal31, and linker-scanning mutagenesis, among other methods well known to those in the art (Sambrook et al., 2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York).

Rational peptide design has been successfully used to increase the stability of enzymes with respect to thermoinactivation and oxidation. For example, the stability of an enzyme was improved by removal of asparagine residues in  $\alpha$ -amylase (Declerck et al., 2000, J. Mol. Biol. 301:1041-1057), the introduction of more rigid structural elements such as proline into  $\alpha$ -amylase (Igarashi et al., 1999, Biosci. Biotechnol. Biochem. 63:1535-1540) and D-xylose isomerase (Zhu et al., 1999, Peptide Eng. 12:635-638). Further, the introduction of additional hydrophobic contacts stabilized 3-isopropylmalate dehydrogenase (Akanuma et al., 1999, Eur. J. Biochem. 260:499-504) and formate dehydrogenase obtained from *Pseudomonas* sp. (Rojkova et al., 1999, FEBS Lett. 445:183-188). The mechanisms

can be carried out in an automatic DNA synthesizer, and the synthetic genes may then be ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al. (1981) Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp. 273-289; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477. Such techniques have been employed in the directed evolution of other peptides (see, for example, Scott et al. (1990) Science 249:386-390; Roberts et al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; as well as U.S. Pat. Nos. 5,223,409, 5,198,346, and 5,096,815).

a. Directed evolution

Peptides useful in the methods of the invention may also be generated using "directed evolution" techniques. In contrast to site directed mutagenesis techniques where knowledge of the structure of the peptide is required, there now exist strategies to generate libraries of mutations from which to obtain peptides with improved properties without knowledge of the structural features of the peptide. These strategies are generally known as "directed evolution" technologies and are different from traditional random mutagenesis procedures in that they involve subjecting the nucleic acid sequence encoding the peptide of interest to recursive rounds of mutation, screening and amplification.

In some "directed evolution" techniques, the diversity in the nucleic acids obtained is generated by mutation methods that randomly create point mutations in the nucleic acid sequence. The point mutation techniques include, but are not limited to, "error-prone PCR<sup>TM</sup>" (Caldwell and Joyce, 1994; PCR Methods Appl. 2: 28-33; and Ke and Madison, 1997, Nucleic Acids Res. 25: 3371-3372), repeated oligonucleotide-directed mutagenesis (Reidhaar-Olson et al., 1991, Methods Enzymol. 208:564-586), and any of the aforementioned methods of random mutagenesis.

Another method of creating diversity upon which directed evolution can act is the use of mutator genes. The nucleic acid of interest is cultured in a mutator cell strain the genome of which typically encodes defective DNA repair genes (U.S. Patent No. 6,365,410; Selifonova et al., 2001, Appl. Environ. Microbiol. 67:3645-3649; Long-McGie et al., 2000, Biotech. Bioeng. 68:121-125; see, Genencor International Inc, Palo Alto CA).

Achieving diversity using directed evolution techniques may also be accomplished using saturation mutagenesis along with degenerate primers (Gene Site Saturation Mutagenesis™, Diversa Corp., San Diego, CA). In this type of saturation mutagenesis, degenerate primers designed to cover the length of the nucleic acid sequence to be diversified are used to prime the polymerase in PCR reactions. In this manner, each codon of a coding sequence for an amino acid may be mutated to encode each of the remaining common nineteen amino acids. This technique may also be used to introduce mutations, deletions and insertions to specific regions of a nucleic acid coding sequence while leaving the rest of the nucleic acid molecule untouched. Procedures for the gene saturation technique are well known in the art, and can be found in U.S. Patent 6,171,820.

#### b. DNA shuffling

Novel peptides useful in the methods of the invention may also be generated using the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling techniques are may be employed to modulate the activities of peptides useful in the invention and may be used to generate peptides having altered activity. See, generally, U.S. Pat. Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Stemmer et al. (1994, Nature 370(6488):389-391); Cramer et al. (1998, Nature 391 (6664):288-291); Zhang et al. (1997, Proc. Natl. Acad. Sci. USA 94(9):4504-4509); Stemmer et al. (1994, Proc. Natl. Acad. Sci. USA 91(22):10747-10751), Patten et al. (1997, Curr. Opinion Biotechnol. 8:724-33); Harayama, (1998, Trends Biotechnol. 16(2):76-82); Hansson, et al., (1999, J. Mol. Biol. 287:265-76); and Lorenzo and Blasco (1998, Biotechniques 24(2):308-13) (each of these patents are hereby incorporated by reference in its entirety).

DNA shuffling involves the assembly of two or more DNA segments by homologous or site-specific recombination to generate variation in the polynucleotide sequence. DNA shuffling has been used to generate novel variations of human immunodeficiency virus type 1 proteins (Pekrun et al., 2002, J. Virol. 76(6):2924-35), triazine hydrolases (Raillard et al. 2001, Chem Biol 8(9):891-898), murine leukemia virus (MLV) proteins (Powell et al. 2000, Nat Biotechnol 18(12):1279-1282), and indoleglycerol phosphate synthase (Merz et al. 2000, Biochemistry 39(5):880-889).

combined with *in vivo* homologous recombination in yeast (Abecassis et al., 2000, Nucleic Acids Res. 28:E88; ). To maximize intergenic recombination, single stranded DNA from complementary strands of each of the nucleic acids are digested with DNase and annealed (Kikuchi et al., 2000, Gene 243:133-137). The blunt ends of two truncated nucleic acids of variable lengths that are linked by a cleavable sequence are then ligated to generate gene fusion without homologous recombination (Sieber et al., 2001, Nat Biotechnol. 19:456-460; Lutz et al., 2001, Nucleic Acids Res. 29:E16; Ostermeier et al., 1999, Nat. Biotechnol. 17:1205-1209; Lutz and Benkovic, 2000, Curr. Opin. Biotechnol. 11:319-324). Recombination between nucleic acids with little sequence homology in common has also been enhanced using exonuclease-mediated blunt-ending of DNA fragments and ligating the fragments together to recombine them (U.S. Patent No. 6,361,974, incorporated herein by reference in its entirety). The invention contemplates the use of each and every variation described above as a means of enhancing the biological properties of any of the peptides and/or enzymes useful in the methods of the invention.

In addition to published protocols detailing directed evolution and gene shuffling techniques, commercial services are now available that will undertake the gene shuffling and selection procedures on peptides of choice. Maxygen (Redwood City, CA) offers commercial services to generate custom DNA shuffled libraries. In addition, this company will perform customized directed evolution procedures including gene shuffling and selection on a peptide family of choice.

Optigenix, Inc. (Newark, DE) offers the related service of plasmid shuffling. Optigenix uses families of genes to obtain mutants therein having new properties. The nucleic acid of interest is cloned into a plasmid in an *Aspergillus* expression system. The DNA of the related family is then introduced into the expression system and recombination in conserved regions of the family occurs in the host. Resulting mutant DNAs are then expressed and the peptide produced therefrom are screened for the presence of desired properties and the absence of undesired properties.

#### c. Screening procedures

Following each recursive round of "evolution," the desired peptides expressed by mutated genes are screened for characteristics of interest. The "candidate" genes are then amplified and pooled for the next round of DNA shuffling. The screening procedure used is

highly dependant on the peptide that is being “evolved” and the characteristic of interest. Characteristics such as peptide stability, biological activity, antigenicity, among others can be selected using procedures that are well known in the art. Individual assays for the biological activity of preferred peptides useful in the methods of the invention are described elsewhere  
5 herein.

#### d. Combinations of techniques

It will be appreciated by the skilled artisan that the above techniques of mutation and selection can be combined with each other and with additional procedures to generate the best possible peptide molecule useful in the methods of the invention. Thus, the invention is not  
10 limited to any one method for the generation of peptides, and should be construed to encompass any and all of the methodology described herein. For example, a procedure for introducing point mutations into a nucleic acid sequence may be performed initially, followed by recursive rounds of DNA shuffling, selection and amplification. The initial introduction of point mutations may be used to introduce diversity into a gene population where it is  
15 lacking, and the following round of DNA shuffling and screening will select and recombine advantageous point mutations.

### III. Glycosidases and Glycotransferases

#### A. Glycosidases

20 Glycosidases are glycosyltransferases that use water as an acceptor molecule, and as such, are typically glycoside-hydrolytic enzymes. Glycosidases can be used for the formation of glycosidic bonds *in vitro* by controlling the thermodynamics or kinetics of the reaction mixture. Even with modified reaction conditions, though, glycosidase reactions can be difficult to work with, and glycosidases tend to give low synthetic yields as a result of the  
25 reversible transglycosylase reaction and the competing hydrolytic reaction.

A glycosidase can function by retaining the stereochemistry at the bond being broken during hydrolysis or by inverting the stereochemistry at the bond being broken during hydrolysis, classifying the glycosidase as either a “retaining” glycosidase or an “inverting” glycosidase, respectively. Retaining glycosidases have two critical carboxylic acid moieties  
30 present in the active site, with one carboxylate acting as an acid/base catalyst and the other as

a nucleophile, whereas with the inverting glycosidases, one carboxylic acid functions as an acid and the other functions as a base.

Methods to determine the activity and linkage specificity of any glycosidase are well known in the art, including a simplified HPLC protocol (Jacob and Scudder, 1994, Methods in Enzymol. 230: 280-300). A general discussion of glycosidases and glycosidase treatment is found in Glycobiology, A Practical Approach, (1993, Fukuda and Kobata eds., Oxford University Press Inc., New York).

Glycosidases useful in the invention include, but are not limited to, sialidase, galactosidase, endoglucanase, mannosidase (i.e.,  $\alpha$  and  $\beta$ , ManI, ManII and ManIII,) xylosidase, fucosidase, *Agrobacterium* sp.  $\beta$ -glucosidase, *Cellulomonas fimi* mannosidase 2A, *Humicola insolens* glycosidase, *Sulfolobus solfataricus* glycosidase and *Bacillus licheniformis* glycosidase.

The choice of fucosidases for use in the invention depends on the linkage of the fucose to other molecules. The specificities of many  $\alpha$ -fucosidases useful in the methods of the invention are well known to those in the art, and many varieties of fucosidase are also commercially available (Glyko, Novato, CA; PROzyme, San Leandro, CA; Calbiochem-Novabiochem Corp., San Diego, CA; among others).  $\alpha$ -Fucosidases of interest include, but are not limited to,  $\alpha$ -fucosidases from *Turbo cornutus*, *Charonia lampas*, *Bacillus fulminans*, *Aspergillus niger*, *Clostridium perfringens*, Bovine kidney (Glyko), chicken liver (Tyagarajan et al., 1996, Glycobiology 6:83-93) and  $\alpha$ -fucosidase II from *Xanthomonas manihotis* (Glyko, PROzyme). Chicken liver fucosidase is particularly useful for removal of core fucose from N-linked glycans.

#### B. Glycosyltransferases

Glycosyltransferases catalyze the addition of activated sugars (donor NDP-sugars), in a step-wise fashion, to a protein, glycopeptide, lipid or glycolipid or to the non-reducing end of a growing oligosaccharide. N-linked glycopeptides are synthesized via a transferase and a lipid-linked oligosaccharide donor Dol-PP-NAG<sub>2</sub>Glc<sub>3</sub>Man<sub>9</sub> in an en block transfer followed by trimming of the core. In this case the nature of the "core" saccharide is somewhat different from subsequent attachments. A very large number of glycosyltransferases are known in the art.

The glycosyltransferase to be used in the present invention may be any as long as it can utilize the modified sugar as a sugar donor. Examples of such enzymes include Leloir pathway glycosyltransferase, such as galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, fucosyltransferase, sialyltransferase, mannosyltransferase, xylosyltransferase, glucurononyltransferase and the like.

For enzymatic saccharide syntheses that involve glycosyltransferase reactions, glycosyltransferase can be cloned, or isolated from any source. Many cloned glycosyltransferases are known, as are their polynucleotide sequences. *See, e.g.,* Taniguchi et al., 2002, Handbook of glycosyltransferases and related genes, Springer, Tokyo.

Glycosyltransferase amino acid sequences and nucleotide sequences encoding glycosyltransferases from which the amino acid sequences can be deduced are also found in various publicly available databases, including GenBank, Swiss-Prot, EMBL, and others.

Glycosyltransferases that can be employed in the methods of the invention include, but are not limited to, galactosyltransferases, fucosyltransferases, glucosyltransferases, N-acetylgalactosaminyltransferases, N-acetylglucosaminyltransferases, glucuronyltransferases, sialyltransferases, mannosyltransferases, glucuronic acid transferases, galacturonic acid transferases, and oligosaccharyltransferases. Suitable glycosyltransferases include those obtained from eukaryotes, as well as from prokaryotes.

DNA encoding glycosyltransferases may be obtained by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic libraries from appropriate cells, or by combinations of these procedures. Screening of mRNA or genomic DNA may be carried out using oligonucleotide probes generated from the glycosyltransferases nucleic acid sequence. Probes may be labeled with a detectable label, such as, but not limited to, a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with known procedures and used in conventional hybridization assays. In the alternative, glycosyltransferases nucleic acid sequences may be obtained by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers being produced from the glycosyltransferases nucleic acid sequence. *See, U.S. Pat. No. 4,683,195 to Mullis et al. and U.S. Pat. No. 4,683,202 to Mullis.*

A glycosyltransferases enzyme may be synthesized in a host cell transformed with a vector containing DNA encoding the glycosyltransferases enzyme. A vector is a replicable

DNA construct. Vectors are used either to amplify DNA encoding the glycosyltransferases enzyme and/or to express DNA which encodes the glycosyltransferases enzyme. An expression vector is a replicable DNA construct in which a DNA sequence encoding the glycosyltransferases enzyme is operably linked to suitable control sequences capable of effecting the expression of the glycosyltransferases enzyme in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

#### 1. Fucosyltransferases

In some embodiments, a glycosyltransferase used in the method of the invention is a fucosyltransferase. Fucosyltransferases are known to those of skill in the art. Exemplary fucosyltransferases include enzymes, which transfer L-fucose from GDP-fucose to a hydroxy position of an acceptor sugar. Fucosyltransferases that transfer from non-nucleotide sugars to an acceptor are also of use in the present invention.

In some embodiments, the acceptor sugar is, for example, the GlcNAc in a Gal $\beta$ (1 $\rightarrow$ 3,4)GlcNAc $\beta$ - group in an oligosaccharide glycoside. Suitable fucosyltransferases for this reaction include the Gal $\beta$ (1 $\rightarrow$ 3,4)GlcNAc $\beta$ 1- $\alpha$ (1 $\rightarrow$ 3,4)fucosyltransferase (FTIII E.C. No. 2.4.1.65), which was first characterized from human milk (see, Palcic, et al., Carbohydrate Res. 190: 1-11 (1989); Prieels, et al., J. Biol. Chem. 256: 10456-10463 (1981); and Nunez, et al., Can. J. Chem. 59: 2086-2095 (1981)) and the Gal $\beta$ (1 $\rightarrow$ 4)GlcNAc $\beta$ - $\alpha$ fucosyltransferases (FTIV, FTV, FTVI) which are found in human serum. FTVII (E.C. No. 2.4.1.65), a sialyl  $\alpha$ (2 $\rightarrow$ 3)Gal $\beta$ ((1 $\rightarrow$ 3)GlcNAc $\beta$  fucosyltransferase, has also been characterized. A recombinant form of the Gal $\beta$ (1 $\rightarrow$ 3,4) GlcNAc $\beta$ - $\alpha$ (1 $\rightarrow$ 3,4)fucosyltransferase has also been characterized (see, Dumas, et al., Bioorg. Med. Letters 1: 425-428 (1991) and Kukowska-Latallo, et al., Genes and Development 4: 1288-1303 (1990)). Other exemplary fucosyltransferases include, for example,  $\alpha$ 1,2



fucosyltransferase (E.C. No. 2.4.1.69). Enzymatic fucosylation can be carried out by the methods described in Mollicone, et al., Eur. J. Biochem. 191: 169-176 (1990) or U.S. Patent No. 5,374,655.

## 2. Galactosyltransferases

In another group of embodiments, the glycosyltransferase is a galactosyltransferase. Exemplary galactosyltransferases include  $\alpha(1,3)$  galactosyltransferases (E.C. No. 2.4.1.151, see, e.g., Dabkowski et al., Transplant Proc. 25:2921 (1993) and Yamamoto et al. Nature 345: 229-233 (1990), bovine (GenBank j04989, Joziassse et al., J. Biol. Chem. 264: 14290-14297 (1989)), murine (GenBank m26925; Larsen et al., Proc. Nat'l. Acad. Sci. USA 86: 8227-8231 (1989)), porcine (GenBank L36152; Strahan et al., Immunogenetics 41: 101-105 (1995)). Another suitable  $\alpha 1,3$  galactosyltransferase is that which is involved in synthesis of the blood group B antigen (EC 2.4.1.37, Yamamoto *et al.*, J. Biol. Chem. 265: 1146-1151 (1990) (human)).

Also suitable for use in the methods of the invention are  $\beta(1,4)$  galactosyltransferases, which include, for example, EC 2.4.1.90 (LacNAc synthetase) and EC 2.4.1.22 (lactose synthetase) (bovine (D'Agostaro et al., Eur. J. Biochem. 183: 211-217 (1989)), human (Masri et al., Biochem. Biophys. Res. Commun. 157: 657-663 (1988)), murine (Nakazawa et al., J. Biochem. 104: 165-168 (1988)), as well as E.C. 2.4.1.38 and the ceramide galactosyltransferase (EC 2.4.1.45, Stahl et al., J. Neurosci. Res. 38: 234-242 (1994)). Other suitable galactosyltransferases include, for example,  $\alpha 1,2$  galactosyltransferases (from e.g., *Schizosaccharomyces pombe*, Chapell et al., Mol. Biol. Cell 5: 519-528 (1994)). For further suitable galactosyltransferases, see Taniguchi et al. (2002, Handbook of Glycosyltransferases and Related Genes, Springer, Tokyo), Guo et al. (2001, Glycobiology, 11(10):813-820), and Breton et al. (1998, J Biochem. 123:1000-1009).

The production of proteins such as the enzyme GalNAc T<sub>I-XIV</sub> from cloned genes by genetic engineering is well known. See, e.g., U.S. Pat. No. 4,761,371. One method involves collection of sufficient samples, then the amino acid sequence of the enzyme is determined by N-terminal sequencing. This information is then used to isolate a cDNA clone encoding a full-length (membrane bound) transferase which upon expression in the insect cell line Sf9 resulted in the synthesis of a fully active enzyme. The acceptor specificity of the enzyme is

then determined using a semiquantitative analysis of the amino acids surrounding known glycosylation sites in 16 different proteins followed by *in vitro* glycosylation studies of synthetic peptides. This work has demonstrated that certain amino acid residues are overrepresented in glycosylated peptide segments and that residues in specific positions surrounding glycosylated serine and threonine residues may have a more marked influence on acceptor efficiency than other amino acid moieties.

### 3. Sialyltransferases

Sialyltransferases are another type of glycosyltransferase that is useful in the recombinant cells and reaction mixtures of the invention. Examples of sialyltransferases that are suitable for use in the present invention include ST3Gal III (e.g., a rat or human ST3Gal III), ST3Gal IV, ST3Gal I, ST6Gal I, ST3Gal V, ST6Gal II, ST6GalNAc I, ST6GalNAc II, and ST6GalNAc III (the sialyltransferase nomenclature used herein is as described in Tsuji et al., *Glycobiology* 6: v-xiv (1996)). An exemplary  $\alpha(2,3)$ sialyltransferase referred to as  $\alpha(2,3)$ sialyltransferase (EC 2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Gal $\beta$ 1 $\rightarrow$ 3Glc disaccharide or glycoside. See, Van den Eijnden et al., *J. Biol. Chem.* 256: 3159 (1981), Weinstein et al., *J. Biol. Chem.* 257: 13845 (1982) and Wen et al., *J. Biol. Chem.* 267: 21011 (1992). Another exemplary  $\alpha 2,3$ -sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of the disaccharide or glycoside. see, Rearick et al., *J. Biol. Chem.* 254: 4444 (1979) and Gillespie et al., *J. Biol. Chem.* 267: 21004 (1992). Further exemplary enzymes include Gal- $\beta$ -1,4-GlcNAc  $\alpha$ -2,6 sialyltransferase (See, Kurosawa et al. *Eur. J. Biochem.* 219: 375-381 (1994)).

Preferably, for glycosylation of carbohydrates of glycopeptides the sialyltransferase will be able to transfer sialic acid to the sequence Gal $\beta$ 1,4GlcNAc-, Gal $\beta$ 1,3GlcNAc-, or Gal $\beta$ 1,3GalNAc-, the most common penultimate sequences underlying the terminal sialic acid on fully sialylated carbohydrate structures (see, Table 7). 2,8-Sialyltransferases capable of transferring sialic acid to  $\alpha 2,3$ Gal $\beta$ 1,4GlcNAc are also useful in the methods of the invention.

Table 7. Sialyltransferases which use the Gal $\beta$ 1,4GlcNAc sequence as an acceptor substrate

Sialyltransferase	Source	Sequence(s) formed	Ref.
ST6Gal I	Mammalian	NeuAc $\alpha$ 2,6Gal $\beta$ 1,4GlcNAc-	1
ST3Gal III	Mammalian	NeuAc $\alpha$ 2,3Gal $\beta$ 1,4GlcNAc- NeuAc $\alpha$ 2,3Gal $\beta$ 1,3GlcNAc-	1
ST3Gal IV	Mammalian	NeuAc $\alpha$ 2,3Gal $\beta$ 1,4GlcNAc- NeuAc $\alpha$ 2,3Gal $\beta$ 1,3GlcNAc-	1
ST6Gal II	Mammalian	NeuAc $\alpha$ 2,6Gal $\beta$ 1,4GlcNAc-	
ST6Gal II	<i>Photobacterium</i>	NeuAc $\alpha$ 2,6Gal $\beta$ 1,4GlcNAc-	2
ST3Gal V	<i>N. meningitides</i> <i>N. gonorrhoeae</i>	NeuAc $\alpha$ 2,3Gal $\beta$ 1,4GlcNAc-	3

1) Goochee et al., Bio/Technology 9: 1347-1355 (1991)

2) Yamamoto et al., J. Biochem. 120: 104-110 (1996)

5 3) Gilbert et al., J. Biol. Chem. 271: 28271-28276 (1996)

An example of a sialyltransferase that is useful in the claimed methods is ST3Gal III, which is also referred to as  $\alpha$ (2,3)sialyltransferase (EC 2.4.99.6). This enzyme catalyzes the transfer of sialic acid to the Gal of a Gal $\beta$ 1,3GlcNAc or Gal $\beta$ 1,4GlcNAc glycoside (see, e.g.,  
10 Wen et al., J. Biol. Chem. 267: 21011 (1992); Van den Eijnden et al., J. Biol. Chem. 256: 3159 (1991)) and is responsible for sialylation of asparagine-linked oligosaccharides in glycopeptides. The sialic acid is linked to a Gal with the formation of an  $\alpha$ -linkage between the two saccharides. Bonding (linkage) between the saccharides is between the 2-position of NeuAc and the 3-position of Gal. This particular enzyme can be isolated from rat liver  
15 (Weinstein et al., J. Biol. Chem. 257: 13845 (1982)); the human cDNA (Sasaki et al. (1993) J. Biol. Chem. 268: 22782-22787; Kitagawa & Paulson (1994) J. Biol. Chem. 269: 1394-1401) and genomic (Kitagawa et al. (1996) J. Biol. Chem. 271: 931-938) DNA sequences are known, facilitating production of this enzyme by recombinant expression. In a preferred embodiment, the claimed sialylation methods use a rat ST3Gal III.

Other exemplary sialyltransferases of use in the present invention include those isolated from *Campylobacter jejuni*, including the  $\alpha(2,3)$ . See, e.g., WO99/49051.

Other sialyltransferases, including those listed in Table 7, are also useful in an economic and efficient large-scale process for sialylation of commercially important glycopeptides. As a simple test to find out the utility of these other enzymes, various amounts of each enzyme (1-100 mU/mg protein) are reacted with asialo- $\alpha_1$  AGP (at 1-10 mg/ml) to compare the ability of the sialyltransferase of interest to sialylate glycopeptides relative to either bovine ST6Gal I, ST3Gal III or both sialyltransferases. Alternatively, other glycopeptides or glycopeptides, or N-linked oligosaccharides enzymatically released from the peptide backbone can be used in place of asialo- $\alpha_1$  AGP for this evaluation. Sialyltransferases with the ability to sialylate N-linked oligosaccharides of glycopeptides more efficiently than ST6Gal I are useful in a practical large-scale process for peptide sialylation (as illustrated for ST3Gal III in this disclosure).

#### 4. Other glycosyltransferases

One of skill in the art will understand that other glycosyltransferases can be substituted into similar transferase cycles as have been described in detail for the sialyltransferase. In particular, the glycosyltransferase can also be, for instance, glucosyltransferases, e.g., Alg8 (Stagljev et al., Proc. Natl. Acad. Sci. USA 91: 5977 (1994)) or Alg5 (Heesen et al., Eur. J. Biochem. 224: 71 (1994)).

N-acetylgalactosaminyltransferases are also of use in practicing the present invention. Suitable N-acetylgalactosaminyltransferases include, but are not limited to,  $\alpha(1,3)$  N-acetylgalactosaminyltransferase,  $\beta(1,4)$  N-acetylgalactosaminyltransferases (Nagata et al., J. Biol. Chem. 267: 12082-12089 (1992) and Smith et al., J. Biol. Chem. 269: 15162 (1994)) and peptide N-acetylgalactosaminyltransferase (Homa et al., J. Biol. Chem. 268: 12609 (1993)). Suitable N-acetylglucosaminyltransferases include GnTI (2.4.1.101, Hull et al., BBRC 176: 608 (1991)), GnTII, GnTIII (Ihara et al., J. Biochem. 113: 692 (1993)), GnTIV, GnTV (Shoreibah et al., J. Biol. Chem. 268: 15381 (1993)) and GnTVI, O-linked N-acetylglucosaminyltransferase (Bierhuizen et al., Proc. Natl. Acad. Sci. USA 89: 9326 (1992)), N-acetylglucosamine-1-phosphate transferase (Rajput et al., Biochem J. 285: 985 (1992)), and hyaluronan synthase.

Mannosyltransferases are of use to transfer modified mannose moieties. Suitable mannosyltransferases include  $\alpha(1,2)$  mannosyltransferase,  $\alpha(1,3)$  mannosyltransferase,  $\alpha(1,6)$  mannosyltransferase,  $\beta(1,4)$  mannosyltransferase, Dol-P-Man synthase, OCh1, and Pmt1 (see, Kornfeld et al., Annu. Rev. Biochem. 54: 631-664 (1985)).

5       Xylosyltransferases are also useful in the present invention. See, for example, Rodgers, et al., Biochem. J., 288:817-822 (1992); and Elbain, et al., U.S. Patent No., 6,168,937.

Other suitable glycosyltransferase cycles are described in Ichikawa et al., JACS 114: 9283 (1992), Wong et al., J. Org. Chem. 57: 4343 (1992), and Ichikawa et al. in

10   CARBOHYDRATES AND CARBOHYDRATE POLYMERS. Yaltami, ed. (ATL Press, 1993).

Prokaryotic glycosyltransferases are also useful in practicing the invention. Such glycosyltransferases include enzymes involved in synthesis of lipooligosaccharides (LOS), which are produced by many gram negative bacteria. The LOS typically have terminal glycan sequences that mimic glycoconjugates found on the surface of human epithelial cells or in host secretions (Preston et al., Critical Reviews in Microbiology 23(3): 139-180 (1996)).  
15   Such enzymes include, but are not limited to, the proteins of the *rfa* operons of species such as *E. coli* and *Salmonella typhimurium*, which include a  $\beta(1,6)$  galactosyltransferase and a  $\beta(1,3)$  galactosyltransferase (see, e.g., EMBL Accession Nos. M80599 and M86935 (*E. coli*); EMBL Accession No. S56361 (*S. typhimurium*)), a glucosyltransferase (Swiss-Prot  
20   Accession No. P25740 (*E. coli*), an  $\beta(1,2)$ -glucosyltransferase (*rfaI*)(Swiss-Prot Accession No. P27129 (*E. coli*) and Swiss-Prot Accession No. P19817 (*S. typhimurium*)), and an  $\beta(1,2)$ -N-acetylglucosaminyltransferase (*rfaK*)(EMBL Accession No. U00039 (*E. coli*). Other glycosyltransferases for which amino acid sequences are known include those that are encoded by operons such as *rfaB*, which have been characterized in organisms such as  
25   *Klebsiella pneumoniae*, *E. coli*, *Salmonella typhimurium*, *Salmonella enterica*, *Yersinia enterocolitica*, *Mycobacterium leprosum*, and the *rhl* operon of *Pseudomonas aeruginosa*.

Also suitable for use in the present invention are glycosyltransferases that are involved in producing structures containing lacto-N-neotetraose, D-galactosyl- $\beta(1,4)$ -N-acetyl-D-glucosaminyl- $\beta(1,3)$ -D-galactosyl- $\beta(1,4)$ -D-glucose, and the P<sup>k</sup> blood group  
30   trisaccharide sequence, D-galactosyl- $\alpha(1,4)$ -D-galactosyl- $\beta(1,4)$ -D-glucose, which have been

identified in the LOS of the mucosal pathogens *Neisseria gonorrhoeae* and *N. meningitidis* (Scholten et al., J. Med. Microbiol. 41: 236-243 (1994)). The genes from *N. meningitidis* and *N. gonorrhoeae* that encode the glycosyltransferases involved in the biosynthesis of these structures have been identified from *N. meningitidis* immunotypes L3 and L1 (Jennings et al., 5 Mol. Microbiol. 18: 729-740 (1995)) and the *N. gonorrhoeae* mutant F62 (Gotshlich, J. Exp. Med. 180: 2181-2190 (1994)). In *N. meningitidis*, a locus consisting of three genes, *lgtA*, *lgtB* and *lgtE*, encodes the glycosyltransferase enzymes required for addition of the last three of the sugars in the lacto-*N*-neotetraose chain (Wakarchuk et al., J. Biol. Chem. 271: 19166-73 (1996)). Recently the enzymatic activity of the *lgtB* and *lgtA* gene product was 10 demonstrated, providing the first direct evidence for their proposed glycosyltransferase function (Wakarchuk et al., J. Biol. Chem. 271(45): 28271-276 (1996)). In *N. gonorrhoeae*, there are two additional genes, *lgtD* which adds  $\beta$ -D-GalNAc to the 3 position of the terminal galactose of the lacto-*N*-neotetraose structure and *lgtC* which adds a terminal  $\alpha$ -D-Gal to the lactose element of a truncated LOS, thus creating the P<sup>k</sup> blood group antigen structure 15 (Gotshlich (1994), *supra.*). In *N. meningitidis*, a separate immunotype L1 also expresses the P<sup>k</sup> blood group antigen and has been shown to carry an *lgtC* gene (Jennings *et al.*, (1995), *supra.*). *Neisseria* glycosyltransferases and associated genes are also described in USPN 5,545,553 (Gotshlich). Genes for  $\alpha$ 1,2-fucosyltransferase and  $\alpha$ 1,3-fucosyltransferase from *Helicobacter pylori* has also been characterized (Martin *et al.*, J. Biol. Chem. 272: 21349-20 21356 (1997)). Also of use in the present invention are the glycosyltransferases of *Campylobacter jejuni* (see, Taniguchi et al., 2002, Handbook of glycosyltransferases and related genes, Springer, Tokyo).

#### B. Sulfotransferases

The invention also provides methods for producing peptides that include sulfated 25 molecules, including, for example sulfated polysaccharides such as heparin, heparan sulfate, carragenen, and related compounds. Suitable sulfotransferases include, for example, chondroitin-6-sulphotransferase (chicken cDNA described by Fukuta et al., J. Biol. Chem. 270: 18575-18580 (1995); GenBank Accession No. D49915), glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 1 (Dixon et al., Genomics 26: 239-241 30 (1995); UL18918), and glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 2 (murine cDNA described in Orellana et al., J. Biol. Chem. 269: 2270-

2276 (1994) and Eriksson et al., J. Biol. Chem. 269: 10438-10443 (1994); human cDNA described in GenBank Accession No. U2304).

### C. Cell-Bound Glycosyltransferases

In another embodiment, the enzymes utilized in the method of the invention are cell-bound glycosyltransferases. Although many soluble glycosyltransferases are known (see, for example, U.S. Pat. No. 5,032,519), glycosyltransferases are generally in membrane-bound form when associated with cells. Many of the membrane-bound enzymes studied thus far are considered to be intrinsic proteins; that is, they are not released from the membranes by sonication and require detergents for solubilization. Surface glycosyltransferases have been identified on the surfaces of vertebrate and invertebrate cells, and it has also been recognized that these surface transferases maintain catalytic activity under physiological conditions. However, the more recognized function of cell surface glycosyltransferases is for intercellular recognition (Roth, 1990, Molecular Approaches to Supracellular Phenomena,).

Methods have been developed to alter the glycosyltransferases expressed by cells. For example, Larsen et al., Proc. Natl. Acad. Sci. USA 86: 8227-8231 (1989), report a genetic approach to isolate cloned cDNA sequences that determine expression of cell surface oligosaccharide structures and their cognate glycosyltransferases. A cDNA library generated from mRNA isolated from a murine cell line known to express UDP-galactose:β-D-galactosyl-1,4-N-acetyl-D-glucosaminide α-1,3-galactosyltransferase was transfected into COS-1 cells. The transfected cells were then cultured and assayed for α 1-3 galactosyltransferase activity.

Francisco et al., Proc. Natl. Acad. Sci. USA 89: 2713-2717 (1992), disclose a method of anchoring β-lactamase to the external surface of *Escherichia coli*. A tripartite fusion consisting of (i) a signal sequence of an outer membrane protein, (ii) a membrane-spanning section of an outer membrane protein, and (iii) a complete mature β-lactamase sequence is produced resulting in an active surface bound β-lactamase molecule. However, the Francisco method is limited only to prokaryotic cell systems and as recognized by the authors, requires the complete tripartite fusion for proper functioning.

#### D. Fusion Enzymes

In other exemplary embodiments, the methods of the invention utilize fusion peptides that have more than one enzymatic activity that is involved in synthesis of a desired glycopeptide conjugate. The fusion peptides can be composed of, for example, a catalytically active domain of a glycosyltransferase that is joined to a catalytically active domain of an accessory enzyme. The accessory enzyme catalytic domain can, for example, catalyze a step in the formation of a nucleotide sugar that is a donor for the glycosyltransferase, or catalyze a reaction involved in a glycosyltransferase cycle. For example, a polynucleotide that encodes a glycosyltransferase can be joined, in-frame, to a polynucleotide that encodes an enzyme involved in nucleotide sugar synthesis. The resulting fusion peptide can then catalyze not only the synthesis of the nucleotide sugar, but also the transfer of the sugar moiety to the acceptor molecule. The fusion peptide can be two or more cycle enzymes linked into one expressible nucleotide sequence. In other embodiments the fusion peptide includes the catalytically active domains of two or more glycosyltransferases. See, for example, U.S. Patent No. 5,641,668. The modified glycopeptides of the present invention can be readily designed and manufactured utilizing various suitable fusion peptides (*see, for example, PCT Patent Application PCT/CA98/01180, which was published as WO 99/31224 on June 24, 1999.*)

#### E. Immobilized Enzymes

In addition to cell-bound enzymes, the present invention also provides for the use of enzymes that are immobilized on a solid and/or soluble support. In an exemplary embodiment, there is provided a glycosyltransferase that is conjugated to a PEG via an intact glycosyl linker according to the methods of the invention. The PEG-linker-enzyme conjugate is optionally attached to solid support. The use of solid supported enzymes in the methods of the invention simplifies the work up of the reaction mixture and purification of the reaction product, and also enables the facile recovery of the enzyme. The glycosyltransferase conjugate is utilized in the methods of the invention. Other combinations of enzymes and supports will be apparent to those of skill in the art.

#### F. Mutagenesis of Glycosyltransferases

The novel forms of the glycosyltransferases, sialyltransferases, sulfotransferases, and any other enzymes used in the method of the invention can be created using any of the



methods described previously, as well as others well known to those in the art. Of particular interest are transferases with altered acceptor specificity and/or donor specificity. Also of interest are enzymes with higher conversion rates and higher stability among others.

The techniques of rational design mutagenesis can be used when the sequence of the peptide is known. Since the sequences as well as many of the tertiary structures of the transferases and glucosidases used in the invention are known, these enzymes are ideal for rational design of mutants. For example, the catalytic site of the enzyme can be mutated to alter the donor and/or acceptor specificity of the enzyme.

The extensive tertiary structural data on the glycosyltransferases and glycosidase hydrolases also make these enzyme idea for mutations involving domain exchanges. Glycosyltransferases and glycosidase hydrolases are modular enzymes (see, Bourne and Henrissat, 2001, Current Opinion in Structural Biology 11:593-600). Glycosyltransferases are divided into two families bases on their structure: GT-A and GT-B. The glycosyltransferases of the GT-A family comprise two dissimilar domains, one involved in nucleotide binding and the other in acceptor binding. Thus, one could conveniently fuse the DNA sequence encoding the domain from one gene in frame with a domain from a second gene to create a new gene that encodes a protein with a new acceptor/donor specificity. Such exchanges of domains could additionally include the carbohydrate modules and other accessory domains.

The techniques of random mutation and/or directed evolution, as described above, may also be used to create novel forms of the glycosyltransferases and glycosidases used in the invention.

#### IV. *In vitro* and *in vivo* expression systems

##### A. Cells for the production of glycopeptides

The action of glycosyltransferases is key to the glycosylation of peptides, thus, the difference in the expression of a set of glycosyltransferases in any given cell type affects the pattern of glycosylation on any given peptide produced in that cell. For a review of host cell dependent glycosylation of peptides, see Kabata and Takasaki, "Structure and Biosynthesis of Cell Surface Carbohydrates," in Cell Surface Carbohydrates and Cell Development, 1991, pp. 1-24, Eds. Minoru Fukuda, CRC Press, Boca Raton, FL.

According to the present disclosure, the type of cell in which the peptide is produced is relevant only with respect to the degree of remodeling required to generate a peptide having desired glycosylation. For example, the number and sequence of enzymatic digestion reactions and the number and sequence of enzymatic synthetic reactions that are required *in vitro* to generate a peptide having desired glycosylation will vary depending on the structure of the glycan on the peptide produced by a particular cell type. While the invention should in no way be construed to be limited to the production of peptides from any one particular cell type including any cell type disclosed herein, a discussion of several cell systems is now presented which establishes the power of the present invention and its independence of the cell type in which the peptides are generated.

In general, and to express a peptide from a nucleic acid encoding it, the nucleic acid must be incorporated into an expression cassette, comprising a promoter element, a terminator element, and the coding sequence of the peptide operably linked between the two. The expression cassette is then operably linked into a vector. Toward this end, adapters or linkers may be employed to join the nucleotide fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous nucleotides, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved. A shuttle vector has the genetic elements necessary for replication in a cell. Some vectors may be replicated only in prokaryotes, or may be replicated in both prokaryotes and eukaryotes. Such a plasmid expression vector will be maintained in one or more replication systems, preferably two replications systems, that allow for stable maintenance within a yeast host cell for expression purposes, and within a prokaryotic host for cloning purposes. Many vectors with diverse characteristics are now available commercially. Vectors are usually plasmids or phages, but may also be cosmids or mini-chromosomes. Conveniently, many commercially available vectors will have the promoter and terminator of the expression cassette already present, and a multi-linker site where the coding sequence for the peptide of interest can be inserted. The shuttle vector containing the expression cassette is then transformed in *E. coli* where it is replicated during cell division to generate a preparation of vector that is sufficient to transform the host cells of the chosen expression system. The above methodology is well known to those in the art, and protocols by which to accomplish can be found Sambrook et al.

(2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York).

The vector, once purified from the cells in which it is amplified, is then transformed into the cells of the expression system. The protocol for transformation depended on the kind of the cell and the nature of the vector. Transformants are grown in an appropriate nutrient medium, and, where appropriate, maintained under selective pressure to insure retention of endogenous DNA. Where expression is inducible, growth can be permitted of the yeast host to yield a high density of cells, and then expression is induced. The secreted, mature heterologous peptide can be harvested by any conventional means, and purified by chromatography, electrophoresis, dialysis, solvent-solvent extraction, and the like.

The techniques of molecular cloning are well-known in the art. Further, techniques for the procedures of molecular cloning can be found in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Glover et al., (1985, DNA Cloning: A Practical Approach, Volumes I and II); Gait et al., (1985, Oligonucleotide Synthesis); Hames and Higgins (1985, Nucleic Acid Hybridization ); Hames and Higgins (1984, Transcription And Translation); Freshney et al., (1986, Animal Cell Culture); Perbal, (1986, Immobilized Cells And Enzymes, IRL Press); Perbal,(1984, A Practical Guide To Molecular Cloning); Ausubel et al. (2002, Current Protocols in Molecular Biology, John Wiley & Sons, Inc.).

#### B. Fungi and yeast

Peptides produced in yeast are glycosylated and the glycan structures present thereon are primarily high mannose structures. In the case of N-glycans, the glycan structures produced in yeast may contain as many as nine or more mannose residues which may or may not contain additional sugars added thereto. An example of the type of glycan on peptides produced by yeast cells is shown in Figure 5, left side. Irrespective of the number of mannose residues and the type and complexity of additional sugars added thereto, N-glycans as components of peptides produced in yeast cells comprise a trimannosyl core structure as shown in Figure 5. When the glycan structure on a peptide produced by a yeast cell is a high mannose structure, it is a simple matter for the ordinary skilled artisan to remove, *in vitro* using available mannosidase enzymes, all of the mannose residues from the molecule except for those that comprise the trimannosyl core of the glycan, thereby generating a peptide

having an elemental trimannosyl core structure attached thereto. Now, using the techniques available in the art and armed with the present disclosure, it is a simple matter to enzymatically add, *in vitro*, additional sugar moieties to the elemental trimannosyl core structure to generate a peptide having a desired glycan structure attached thereto. Similarly, when the peptide produced by the yeast cell comprises a high mannose structure in addition to other complex sugars attached thereto, it is a simple matter to enzymatically cleave off all of the additional sugars, including extra mannose residues, to arrive at the elemental trimannosyl core structure. Once the elemental trimannosyl core structure is produced, generation of a peptide having desired glycosylation is possible following the directions provided herein.

By "yeast" is intended ascosporeogenous yeasts (Endomycetales), basidiosporeogenous yeasts, and yeast belonging to the Fungi Imperfecti (Blastomycetes). The ascosporeogenous yeasts are divided into two families, *Spermophthoraceae* and *Saccharomycetaceae*. The later is comprised of four subfamilies, *Schizosaccharomycoideae* (e.g., genus *Schizosaccharomyces*), *Nadsonioideae*, *Lipomycoideae*, and *Saccharomycoideae* (e.g., genera *Pichia*, *Kluyveromyces*, and *Saccharomyces*). The basidiosporeogenous yeasts include the genera *Leucosporidium*, *Rhodosporeidium*, *Sporidiobolus*, *Filobasidium*, and *Filobasidiella*. Yeast belonging to the Fungi Imperfecti are divided into two families, *Sporobolomycetaceae* (e.g., genera *Sporobolomyces*, *Bullera*) and *Cryptococcaceae* (e.g., genus *Candida*). Of particular interest to the present invention are species within the genera *Saccharomyces*, *Pichia*, *Aspergillus*, *Trichoderma*, *Kluyveromyces*, especially *K. lactis* and *K. drosophilum*, *Candida*, *Hansenula*, *Schizosaccharomyces*, *Yarrowia*, and *Chrysosporium*. Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in Skinner et al., eds. 1980) *Biology and Activities of Yeast* (Soc. App. Bacteriol. Symp. Series No. 9).

In addition to the foregoing, those of ordinary skill in the art are presumably familiar with the biology of yeast and the manipulation of yeast genetics. See, for example, Bacila et al., eds. (1978, *Biochemistry and Genetics of Yeast*, Academic Press, New York); and Rose and Harrison. (1987, *The Yeasts* (2<sup>nd</sup> ed.) Academic Press, London). Methods of introducing exogenous DNA into yeast hosts are well known in the art. There are a wide variety of methods for transformation of yeast. Spheroplast transformation is taught by Hinnen et al

(1978, Proc. Natl. Acad. Sci. USA 75:1919-1933); Beggs, (1978, Nature 275(5676):104-109); and Stinchcomb et al., (EPO Publication No. 45,573; herein incorporated by reference), Electroporation is taught by Becker and Gaurante, (1991, Methods Enzymol. 194:182-187), Lithium acetate is taught by Gietz et al. (2002, Methods Enzymol. 350:87-96) and Mount et al. (1996, Methods Mol Biol. 53:139-145). For a review of transformation systems of non-*Saccharomyces* yeasts, see Wang et al. (Crit Rev Biotechnol. 2001;21(3):177-218). For general procedures on yeast genetic engineering, see Barr et al., (1989, Yeast genetic engineering, Butterworths, Boston).

In addition to wild-type yeast and fungal cells, there are also strains of yeast and fungi that have been mutated and/or selected to enhance the level of expression of the exogenous gene, and the purity, the post-translational processing of the resulting peptide, and the recovery and purity of the mature peptide. Expression of an exogenous peptide may also be direct to the cell secretory pathway, as illustrated by the expression of insulin (see (Kjeldsen, 2000, Appl. Microbiol. Biotechnol. 54:277-286, and references cited therein). In general, to cause the exogenous peptide to be secreted from the yeast cell, secretion signals derived from yeast genes may be used, such as those of the genes of the killer toxin (Stark and Boyd, 1986, EMBO J. 5:1995-2002) or of the alpha pheromone (Kurjan and Herskowitz, 1982, Cell 30:933; Brake et al., 1988, Yeast 4:S436).

Regarding the filamentous fungi in general, methods for genetic manipulation can be found in Kinghorn and Turner (1992, Applied Molecular Genetics of Filamentous Fungi, Blackie Academic and Professional, New York). Guidance on appropriate vectors can be found in Martinelli and Kinghorn (1994, Aspergillus : 50 years, Elsevier, Amsterdam).

### 1. *Saccharomyces*

In *Saccharomyces*, suitable yeast vectors for use producing a peptide include YRp7 (Struhl et al., Proc. Natl. Acad. Sci. USA 76: 1035-1039, 1978), YEpl3 (Broach et al., Gene 8: 121-133, 1979), POT vectors (Kawasaki et al, U.S. Pat. No. 4,931,373, which is incorporated by reference herein), pJDB249 and pJDB219 (Beggs, Nature 275:104-108, 1978) and derivatives thereof. Preferred promoters for use in yeast include promoters for yeast glycolytic gene expression (Hitzeman et al., J. Biol. Chem. 255: 12073-12080, 1980; Alber and Kawasaki, J. Mol. Appl. Genet. 1: 419-434, 1982; Kawasaki, U.S. Pat. No.

4,599,311) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals, Hollaender et al., (eds.), p. 355, Plenum, New York, 1982; Ammerer, Meth. Enzymol. 101: 192-201, 1983), and the ADH2-4<sup>c</sup> promoter (Russell et al., Nature 304: 652-654, 1983; Irani and Kilgore, U.S. patent application Ser. No. 07/784,653, CA 1,304,020 and EP 284 044, which are incorporated herein by reference). The expression units may also include a transcriptional terminator. A preferred transcriptional terminator is the TPI1 terminator (Alber and Kawasaki, *ibid.*).

Examples of such yeast-bacteria shuttle vectors include Yep24 (Botstein et al. (1979) Gene 8:17-24; pC1 (Brake et al. (1984) Proc. Natl. Acad. Sci. USA 81:4642-4646), and Yrp17 (Stnichomb et al. (1982) J. Mol. Biol. 158:157). Additionally, a plasmid expression vector may be a high or low copy number plasmid, the copy number generally ranging from about 1 to about 200. In the case of high copy number yeast vectors, there will generally be at least 10, preferably at least 20, and usually not exceeding about 150 copies of the vector in a single host. Depending upon the heterologous peptide selected, either a high or low copy number vector may be desirable, depending upon the effect of the vector and the recombinant peptide on the host. See, for example, Brake et al. (1984) Proc. Natl. Acad. Sci. USA 81:4642-4646. DNA constructs of the present invention can also be integrated into the yeast genome by an integrating vector. Examples of such vectors are known in the art. See, for example, Botstein et al. (1979) Gene 8:17-24.

The selection of suitable yeast and other microorganism hosts for the practice of the present invention is within the skill of the art. Of particular interest are the *Saccharomyces* species *S. cerevisiae*, *S. carlsbergensis*, *S. diastaticus*, *S. douglasii*, *S. kluyveri*, *S. norbensis*, and *S. oviformis*. When selecting yeast host cells for expression of a desired peptide, suitable host cells may include those shown to have, inter alia, good secretion capacity, low proteolytic activity, and overall vigor. Yeast and other microorganisms are generally available from a variety of sources, including the Yeast Genetic Stock Center, Department of Biophysics and Medical Physics, University of California, Berkeley, Calif.; and the American Type Culture Collection, Manassas VA. For a review, see Strathern et al., eds. (1981, The Molecular Biology of the Yeast *Saccharomyces*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.)

Methods of introducing exogenous DNA into yeast hosts are well known in the art.

## 2. Pichia

The use of *Pichia methanolica* as a host cell for the production of recombinant peptides is disclosed in PCT Applications WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565. DNA molecules for use in transforming *P. methanolica* are commonly prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For peptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (AUG1 or AUG2). Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes, as well as those disclosed in U.S. Patent No. 5,252,726. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. A preferred selectable marker for use in *Pichia methanolica* is a *P. methanolica* ADE2 gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), which allows ade2 host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, host cells in which both methanol utilization genes (AUG1 and AUG2) are deleted are preferred. For production of secreted peptides, host cells deficient in vacuolar protease genes (PEP4 and PRB1) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a peptide of interest into *P. methanolica* cells. It is preferred to transform *P. methanolica* cells by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (t) of from 1 to 40 milliseconds, most preferably about 20 milliseconds. For a review of the use of *Pichia pastoris* for large-scale production of antibody fragments, see Fischer et al., (1999, Biotechnol Appl Biochem. 30 ( Pt 2):117-120).

## 3. Aspergillus

Methods to express peptides in *Aspergillus* spp. are well known in the art, including but not limited to those described in Carrez et al., 1990, Gene 94:147-154; Contreras, 1991, Bio/Technology 9:378-381; Yelton et al., 1984, Proc. Natl. Acad. Sci. USA 81:1470-1474; Tilburn et al., 1983, Gene 26:205-221; Kelly and. Hynes, 1985, EMBO J. 4:475-479; Ballance et al., 1983, Biochem. Biophys. Res. Comm. 112:284-289; Buxton et al., 1985,

Gene 37:207-214, and U.S. Pat. No. 4,935,349, incorporated by reference herein in its entirety. Examples of promoters useful in *Aspergillus* are found in U.S. Patent No. 5,252,726. Strains of *Aspergillus* useful for peptide expression are found in U.S. Patent No. 4,935,349. Commercial production of exogenous peptides is available from Novoenzymes for *Aspergillus niger* and *Aspergillus oryzae*.

#### 4. *Trichoderma*

*Trichoderma* has certain advantages over other species of recombinant host cells for expression of desired peptides. This organism is easy to grow in large quantities and it has the ability to glycosylate and efficiently secrete high yields of recombinant mammalian peptides into the medium, making isolation of the peptide relatively easy. In addition, the glycosylation pattern on expressed peptides is more similar to that on human peptides than peptides expressed in other systems. However, there are still differences in the glycan structures on expressed peptides from these cells. For example, terminal sialic acid residues are important to the therapeutic function of a peptide in a mammalian system, since the presence of these moieties at the end of the glycan structure impedes peptide clearance from the mammalian bloodstream. The mechanism behind the increased biologic half-life of sialylated molecules is believed to lie in their decreased recognition by lectins (Drickamer, 1988, J. Biol. Chem. 263:9557-9560). However, in general fungal cells do not add terminal sialic acid residues to glycans on peptides, and peptides synthesized in fungal cells are therefore asialic. According to the present invention, this deficiency can be remedied using the *in vitro* glycan remodeling methods of the invention described in detail elsewhere herein.

*Trichoderma* species useful as hosts for the production of peptides to be remodeled include *T. reesei*, such as QM6a, ALKO2442 or CBS383.78 (Centraalbureau voor Schimmelcultures, Oosterstraat 1, PO Box 273, 3740 AG Baarn, The Netherlands, or, ATCC13631 (American Type Culture Collection, Manassas VA, 10852, USA, type); *T. viride* (such as CBS189.79 (det. W. Gams); *T. longibrachiatum*, such as CBS816.68 (type); *T. pseudokoningii* (such as MUCL19358; Mycotheque de l'Universite Catholique de Louvain); *T. saturnisporum* CBS330.70 (type); *T. harzianum* CBS316.31 (det. W. Gams); *T. virgatum* (*T. pseudokoningii*) ATCC24961. Most preferably, the host is *T. reesei* and more preferably, it is *T. reesei* strains QM9414 (ATCC 26921), RUT-C-30 (ATCC 56765), and



highly productive mutants such as VTT-D-79125, which is derived from QM9414 (Nevalainen, Technical Research Centre of Finland Publications 26, (1985), Espoo, Finland).

The transformation of *Trichoderma* with DNA is performed using any technique known in the art, including that taught in European patent No. EP0244234, Harkki (1989, Bio/Technology 7:596-601) and Uusitalo (1991, J. Biotech. 17:35-50). Culture of *Trichoderma* is supported by previous extensive experience in industrial scale fermentation techniques; for example, see Finkelstein, 1992, Biotechnology of Filamentous Fungi: Technology and Products, Butterworth-Heinemann, publishers, Stoneham, Mass.

#### 5. *Kluyveromyces*

Yeast belonging to the genus *Kluyveromyces* have been used as host organisms for the production of recombinant peptides. Peptides produced by this genus of yeast are, in particular, chymosin (European Patent 96 430), thaumatin (European Patent 96 910), albumin, interleukin-1 $\beta$ , TPA, TIMP (European Patent 361 991) and albumin derivatives having a therapeutic function (European Patent 413 622). Species of particular interest in the genus *Kluyveromyces* include *K. lactis*.

Methods of expressing recombinant peptides in *Kluyvermyces* spp. are well known in the art. Vectors for the expression and secretion of human recombinant peptides in *Kluyvermyces* are known in the art (Yeh, J. Cell. Biochem. Suppl. 14C:68, Abst. H402; Fleer, 1990, Yeast 6 (Special Issue):S449) as are procedures for transformation and expression of recombinant peptides (Ito et al., 1983, J. Bacteriol. 153:163-168; van den Berg, 1990, Bio/Technology 8:135-139; U.S. Patent No. 5,633,146, WO8304050A1, EP0096910, EP0241435, EP0301670, EP0361991, all of which are incorporated by reference herein in their entirety). For a review of genetic manipulation of *Kluyveromyces lactis* linear DNA plasmids by gene targeting and plasmid shuffles, see Schaffrath et al. (1999, FEMS Microbiol Lett. 178(2):201-210).

#### 6. *Chrysosporium*

The fungal genus *Chrysosporium* has recently been used to expression of foreign recombinant peptides. A description of the procedures by which one of skill in the art can use *Chrysosporium* can be used to express foreign peptides is found in WO 00/20555 (incorporated by reference herein in its entirety). Species particularly suitable for expression

system include, but are not limited to, *C. botryoides*, *C. carmichaelii*, *C. crassitunicatum*, *C. europae*, *C. evolceannui*, *F. fastidium*, *C. filiforme*, *C. gerogiae*, *C. globiferum*, *C. globiferum* var. *articulatum*, *C. globiferum* var. *niveum*, *C. hirundo*, *C. hispanicum*, *C. holmii*, *C. indicum*, *C. inops*, *C. keratinophilum*, *C. kreiselii*, *C. kuzurovianum*, *C. lignorum*, *C. lobatum*, *C. lucknowense*, *C. lucknowense* Garg 2TK, *C. medium*, *C. medium* var. *spissescens*, *C. mephiticum*, *C. merdarium*, *C. merdarium* var. *roseum*, *C. minor*, *C. pannicola*, *C. parvum*, *C. parvum* var. *crescens*, *C. pilosum*, *C. peodomerderium*, *C. pyriformis*, *C. queenslandicum*, *C. sigleri*, *C. sulfureum*, *C. synchronum*, *C. tropicum*, *C. undulatum*, *C. vallenarens*, *C. vespertilium*, and *C. zonatum*.

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### 7. Others

Methods for transforming *Schwanniomyces* are disclosed in European Patent 394 538. Methods for transforming *Acremonium chrysogenum* are disclosed by U.S. Pat. No. 5,162,228. Methods for transforming *Neurospora* are disclosed by U.S. Pat. No. 4,486,533. Also known is an expression system specifically for *Schizosaccharomyces pombe* (European Patent 385 391). General methods for expressing peptides in fission yeast, *Schizosaccharomyces pombe* can be found in Giga-Hama and Kumagai (1997, Foreign gene expression in fission yeast : *Schizosaccharomyces pombe*, Springer, Berlin).

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### C. Mammalian systems

As discussed above, mammalian cells typically produce a heterogeneous mixture of N-glycan structures which vary with respect to the number and arrangement of additional sugars attached to the trimannosyl core. Typically, mammalian cells produce peptides having a complex glycan structure, such as that shown in Figure 4, right side. Using the methods of the present invention, a peptide produced in a mammalian cell may be remodeled *in vitro* to generate a peptide having desired glycosylation by first identifying the primary glycan structure and then determining which sugars must be removed in order to remodel the glycan structure. As discussed herein, the sugars to be removed will determine which cleavage enzymes will be used and thus, the precise steps of the remodeling process will vary depending on the primary glycan structure used as the initial substrate. A sample scheme for remodeling a glycan structure commonly produced in mammalian cells is shown in Figure 3.

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The N-glycan biosynthetic pathway in mammalian cells has been well characterized (reviewed in Moremen, 1994, *Glycobiology* 4:113-125). Many of the enzymes necessary for glycan synthesis have been identified, and mutant cell lines defective in this enzymatic pathway have been isolated including the Chinese hamster ovary (CHO) cell lines Lec23 (defective in alpha-glucosidase I) and Lec18 (novel GlcNAc-TVIII). The glycosylation pattern of peptides produced by these mutant cells is altered relative to normal CHO cells. As discussed herein, the glycosylation defects in these and other mutant cells can be exploited for the purposes of producing a peptide that lacks a complex glycan structure. For example, peptides produced by Lec23 cells lack sialic acid residues, and thus require less enzymatic manipulation in order to reduce the glycan structure to an elemental trimannosyl core or to Man3GlcNAc4. Thus, peptides produced in these cells can serve as preferred substrates for glycan remodeling. One of ordinary skill in the art could isolate or identify other glycosylation-defective cell lines based on known methods, for example the method described in Stanley et al., 1990, *Somatic Cell Mol. Genet.*, 16: 211-223. Use of glycosylation-defective cell lines, those identified and as yet unidentified, is included in the invention for the purpose of generating preferred peptide substrates for the remodeling processes described herein.

Expression vectors useful for expressing exogenous peptides in mammalian cells are numerous, and are well known to those in the art. Many mammalian expression vectors are now commercially available from companies, including Novagen, Inc (Madison, WI), Gene Therapy Systems (San Diego, CA), Promega (Madison, WI), ClonTech Inc. (Palo Alto, CA), and Stratagene (La Jolla, CA), among others.

There are several mammalian cell lines that are particularly adept at expressing exogenous peptides. Typically mammalian cell lines originate from tumor cells extracted from mammals that have become immortalized, that is to say, they can replicate in culture essentially indefinitely. These cell lines include, but are not limited to, CHO (Chinese hamster ovary, e.g. CHO-K1; ATCC No. CCL 61) and variants thereof, NS0 (mouse myeloma), BNK, BHK 570 (ATCC No. CRL 10314), BHK (ATCC No. CRL 1632), Per.C6<sup>TM</sup> (immortalized human cells, Crucell N.V., Leiden, The Netherlands), COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), HEK 293, mouse L cells, T lymphoid cell lines, BW5147 cells and MDCK (Madin-Darby canine kidney), HeLa (human), A549 (human

lung carcinoma), 293 (ATCC No. CRL 1573; Graham et al., 1977, Gen. Virol. 36:59-72), BGMK (Buffalo Green Monkey kidney), Hep-2 (human epidermoid larynx carcinoma), LLC-MK<sub>2</sub> (African Green Monkey Kidney), McCoy, NCI-H292 (human pulmonary mucoepidermoid carcinoma tube), RD (rhabdomyosarcoma), Vero (African Green Monkey kidney), HEL (human embryonic lung), Human Fetal Lung-Chang, MRC5 (human embryonic lung), MRHF (human foreskin), and WI-38 (human embryonic lung). In some cases, the cells in which the therapeutic peptide is expressed may be cells derived from the patient to be treated, or they may be derived from another related or unrelated mammal. For example, fibroblast cells may be isolated from the mammal's skin tissue, and cultured and transformed *in vitro*. This technology is commercially available from Transkaryotic Therapies, Inc. (Cambridge, MA). Almost all currently used cell lines are available from the American Type Culture Collection (ATCC, Manassas, VA) and BioWhittaker (Walkersville, Maryland).

Mammalian cells may be transformed with DNA using any one of several techniques that are well known to those in the art. Such techniques include, but are not limited to, calcium phosphate transformation (Chen and Okayama, 1988 ; Graham and van der Eb, 1973; Corsaro and Pearson, 1981, Somatic Cell Genetics 7:603), Diethylaminoethyl (DEAE)-dextran transfection (Fujita et al., 1986; Lopata et al., 1984; Selden et al., 1986, ), electroporation (Neumann et al., 1982, ; Potter, 1988, ; Potter et al., 1984, ; Wong and Neuman, 1982 ), cationic lipid reagent transfection (Elroy-Stein and Moss, 1990; Feigner et al., 1987; Rose et al., 1991; Whitt et al., 1990; Hawley-Nelson et al., 1993, Focus 15:73; Ciccarone et al., 1993, Focus 15:80), retroviral (Cepko et al., 1984; Miller and Baltimore, 1986; Pear et al., 1993; Austin and Cepko, 1990; Bodine et al., 1991; Fekete and Cepko, 1993; Lemischka et al., 1986; Turner et al., 1990; Williams et al., 1984; Miller and Rosman, 1989, BioTechniques 7:980-90; Wang and Finer, 1996, Nature Med. 2:714-6), polybrene (Chaney et al, 1986; Kawai and Nishizawa, 1984), microinjection (Capecchi, 1980), and protoplast fusion (Rassoulzadegan et al., 1982; Sandri-Goldin et al., 1981; Schaffer, 1980), among others. In general, see Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York) and Ausubel et al. (2002, Current Protocols in Molecular Biology, John Wiley & Sons, New York) for transformation techniques.

Recently the baculovirus system, popular for transformation of insect cells, has been adapted for stable transformation of mammalian cells (see, for review, Koat and Condreay, 2002, Trends Biotechnol. 20:173-180, and references cited therein). The production of recombinant peptides in cultured mammalian cells is disclosed, for example, in U.S. Pat. Nos. 4,713,339, 4,784,950; 4,579,821; and 4,656,134. Several companies offer the services of transformation and culture of mammalian cells, including Cell Trends, Inc. (Middletown, MD). Techniques for culturing mammalian cells are well known in the art, and further found in Hauser et al. (1997, Mammalian Cell Biotechnology, Walter de Gruyter, Inc., Hawthorne, NY), and Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor and references cited therein.

#### D. Insect

Insect cells and in particular, cultured insect cells, express peptides having N-linked glycan structures that are rarely sialylated and usually comprise mannose residues which may or may not have additional fucose residues attached thereto. Examples of the types of glycan structures present on peptides produced in cultured insect cells are shown in Figure 7, and mannose glycans thereof.

Baculovirus-mediated expression in insect cells has become particularly well-established for the production of recombinant peptides (Altmann et al., 1999, Glycoconjugate J. 16:109-123). With regard to peptide folding and post-translational processing, insect cells are second only to mammalian cell lines. However, as noted above, N-glycosylation of peptides in insect cells differs in many respects from N-glycosylation in mammalian cells particularly in that insect cells frequently generate truncated glycan structures comprising oligosaccharides containing just three or sometimes only two mannose residues. These structures may be additionally substituted with fucose residues.

According to the present invention, a peptide produced in an insect cell may be remodeled *in vitro* to generate a peptide with desired glycosylation by first optionally removing any substituted fucose residues using an appropriate fucosidase enzyme. In instances where the peptide comprises an elemental trimannosyl core structure following the removal of fucose residues, then all that is required is the *in vitro* addition of the appropriate sugars to the trimannosyl core structure to generate a peptide having desired glycosylation. In instances when the peptide might contain only two mannose residues in the glycan

structure following removal of any fucose residues, a third mannose residue may be added using a mannosyltransferase enzyme and a suitable donor molecule such as GDP-mannose, and thereafter the appropriate residues are added to generate a peptide having desired glycosylation.

5           Protocols for the use of baculovirus to transform insect cells are well known to those in the art. Several books have been published which provide the procedures to use the baculovirus system to express peptides in insect cells. These books include, but are not limited to, Richardson (Baculovirus Expression Protocols, 1998, Methods in Molecular Biology, Vol 39, Humana Pr), O'Reilly et al. (1994, Baculovirus Expression Vectors : A  
10   Laboratory Manual, Oxford Univ Press), and King and Possee (1992, The Baculovirus Expression System : A Laboratory Guide, Chapman & Hall). In addition, there are also publications such as Lucklow (1993, Curr. Opin. Biotechnol. 4:564-572) and Miller (1993, Curr. Opin. Genet. Dev. 3:97-101).

          Many patents have also been issued that related to systems for baculoviral expression  
15   of foreign proteins. These patents include, but are not limited to, U.S. Patent No. 6,210,966 (Culture medium for insect cells lacking glutamine an contains ammonium salt), U.S. Patent No. 6,090,584 (Use of BVACs (BaculoVirus Artificial Chromosomes) to produce recombinant peptides), U.S. Patent No. 5,871,986 (Use of a baculovirus to express a recombinant nucleic acid in a mammalian cell), U.S. Patent No. 5,759,809 (Methods of  
20   expressing peptides in insect cells and methods of killing insects), U.S. Patent No. 5,753,220 (Cysteine protease gene defective baculovirus, process for its production, and process for the production of economic peptide by using the same), U.S. Patent No. 5,750,383 (Baculovirus cloning system), U.S. Patent No. 5,731,182 (Non-mammalian DNA virus to express a recombinant nucleic acid in a mammalian cell), U.S. Patent No. 5,728,580 (Methods and  
25   culture media for inducing single cell suspension in insect cell lines), U.S. Patent No. 5,583,023 (Modified baculovirus, its preparation process and its application as a gene expression vector), U.S. Patent No. 5,571,709 (Modified baculovirus and baculovirus expression vectors), U.S. Patent No. 5,521,299 (Oligonucleotides for detection of baculovirus infection), U.S. Patent No. 5,516,657 (Baculovirus vectors for expression of secretory and  
30   membrane-bound peptides), U.S. Patent No. 5,475,090 (Gene encoding a peptide which enhances virus infection of host insects), U.S. Patent No. 5,472,858 (Production of

recombinant peptides in insect larvae), U.S. Patent No. 5,348,886 (Method of producing recombinant eukaryotic viruses in bacteria), U.S. Patent No. 5,322,774 (Prokaryotic leader sequence in recombinant baculovirus expression system), U.S. Patent No. 5,278,050 (Method to improve the efficiency of processing and secretion of recombinant genes in insect systems), U.S. Patent No. 5,244,805 (Baculovirus expression vectors), U.S. Patent No. 5,229,293 (Recombinant baculovirus), U.S. Patent No. 5,194,376 (Baculovirus expression system capable of producing recombinant peptides at high levels), U.S. Patent No. 5,179,007 (Method and vector for the purification of recombinant peptides), U.S. Patent No. 5,169,784 (Baculovirus dual promoter expression vector), U.S. Patent No. 5,162,222 (Use of baculovirus early promoters for expression of recombinant nucleic acids in stably transformed insect cells or recombinant baculoviruses), U.S. Patent No. 5,155,037 (Insect signal sequences useful to improve the efficiency of processing and secretion of recombinant nucleic acids in insect systems), U.S. Patent No. 5,147,788 (Baculovirus vectors and methods of use), U.S. Patent No. 5,110,729 (Method of producing peptides using baculovirus vectors in cultured cells), U.S. Patent No. 5,077,214 (Use of baculovirus early promoters for expression of recombinant genes in stably transformed insect cells), U.S. Patent No. 5,023,328 (Lepidopteran AKH signal sequence), and U.S. Patent Nos. 4,879,236 and 4,745,051 (Method for producing a recombinant baculovirus expression vector). All of the aforementioned patents are incorporated in their entirety by reference herein.

Insect cell lines of several different species origin are currently being used for peptide expression, and these lines are well known to those in the art. Insect cell lines of interest include, but are not limited to, dipteran and lepidopteran insect cells in general, Sf9 and variants thereof (fall armyworm *Spodoptera frugiperda*), *Estigmene acrea*, *Trichoplusia ni*, *Bombyx mori*, *Malacosoma disstri*, drosophila lines Kc1 and SL2 among others, and mosquito.

#### E. Plants

Plant cells as peptide producers present a different set of issues. While N-linked glycans produced in plants comprise a trimannosyl core structure, this pentasaccharide backbone may comprise several different additional sugars as shown in Figure 6. For example, in one instance, the trimannosyl core structure is substituted by a  $\beta$ 1,2 linked xylose

residue and an  $\alpha$ 1,3 linked fucose residue. In addition, plant cells may also produce a Man5GlcNAc2 structure. Peptides produced in plant cells are often highly antigenic as a result of the presence of the core  $\alpha$ 1,3 fucose and xylose on the glycan structure, and are rapidly cleared from the blood stream when introduced into a mammal due to the absence of terminal sialic acid residues. Therefore, unless these peptides are remodeled using the methods provided herein, they are generally considered to be unsuitable as therapeutic agents in mammals. While some monoclonal antibodies expressed in plant cells were found to be non-immunogenic in mouse, it is likely that the glycan chains were not immunogenic because they were buried in the Fc region in these antibodies (Chargelegue et al., 2000, Transgenic Res. 9(3):187-194).

Following the directions provided herein, it is now possible to generate a peptide produced in a plant cell wherein an increased number of the glycan structures present thereon comprise an elemental trimannosyl core structure, or a Man3GlcNAc4 structure. This is accomplished by cleaving off any additional sugars *in vitro* using a combination of appropriate glycosidases, including fucosidases, until the elemental trimannosyl core structure or the Man3GlcNAc4 structure is arrived at. These cleavage reactions should also include removal of any fucose or xylose residues from the structures in order to diminish the antigenicity of the final peptide when introduced into a mammal. Plant cells having mutations that inhibit the addition of fucose and xylose residues to the trimannosyl core structure are known in the art (von Schaewen et al., 1993, Plant Physiology 102:1109-1118). The use of these cells to produce peptides having glycans which lack fucose and xylose is contemplated by the invention. Upon production of the elemental trimannosyl core or Man3GlcNAc4 structure, additional sugars may then be added thereto to arrive at a peptide having desired glycosylation that is therefore suitable for therapeutic use in a mammal.

Transgenic plants are considered by many to be the expression system of choice for pharmaceutical peptides. Potentially, plants can provide a cheaper source of recombinant peptides. It has been estimated that the production costs of recombinant peptides in plants could be between 10 to 50 times lower than that of producing the same peptide in *E. coli*. While there are slight differences in the codon usage in plants as compared to animals, these can be compensated for by adjusting the recombinant DNA sequences (see, Kusnadi et al., 1997, Biotechnol. Bioeng. 56:473-484; Khoudi et al., 1999, Biotechnol. Bioeng. 135-143;



Hood et al., 1999, Adv. Exp. Med. Biol. 464:127-147). In addition, peptide synthesis, secretion and post-translational modification are very similar in plants and animals, with only minor differences in plant glycosylation (see, Fischer et al., 2000, J. Biol. Regul. Homest. Agents 14: 83-92). Then, products from transgenic plants are also less likely to be  
5 contaminated by animal pathogens, microbial toxins and oncogenic sequences.

The expression of recombinant peptides in plant cells is well known in the art. In addition to transgenic plants, peptides can also produced in transgenic plant cell cultures (Lee et al., 1997, Mol. Cell. 7:783-787), and non-transgenic plants inoculated with recombinant plant viruses. Several books have been published that describe protocols for the genetic  
10 transformation of plant cells: Potrykus (1995, Gene transfer to plants, Springer, New York), Nickoloff (1995, Plant cell electroporation and electrofusion protocols, Humana Press, Totowa, New York) and Draper (1988, Plant genetic transformation, Oxford Press, Boston).

Several methods are currently used to stably transform plant cells with recombinant genetic material. These methods include, but are not limited to, *Agrobacterium*  
15 transformation (Bechtold and Pelletier, 1998; Escudero and Hohn, 1997; Hansen and Chilton, 1999; Touraev et al., 1997), biolistics (microprojectiles) (Finer et al., 1999; Hansen and Chilton, 1999; Shilito, 1999), electroporation of protoplasts (Fromm et al., 1985, Ou-Lee et al., 1986; Rhodes et al., 1988; Saunders et al., 1989; Trick et al., 1997), polyethylene glycol treatment (Shilito, 1999; Trick et al., 1997), *in planta* microinjection (Leduc et al., 1996;  
20 Zhou et al., 1983), seed imbibition (Trick et al., 1997), laser beam (1996), and silicon carbide whiskers (Thompson et al., 1995; U.S. Patent Appln. No. 20020100077, incorporated by reference herein in its entirety).

Many kinds of plants are amenable to transformation and expression of exogenous peptides. Plants of particular interest to express the peptides to be used in the remodeling  
25 method of the invention include, but are not limited to, *Arabidopsis thaliana*, rapeseed (*Brassica* spp.; Ruiz and Blumwald, 2002, Planta 214:965-969), soybean (*Glycine max*), sunflower (*Helianthus unnuus*), oil palm (*Elaeis guineensis*), groundnut (peanut, *Arachis hypogaea*; Deng et al., 2001, Cell. Res. 11:156-160), coconut (*Cocos nucifera*), castor (*Ricinus communis*), safflower (*Carthamus tinctorius*), mustard (*Brassica* spp. and *Sinapis alba*), coriander, (*Coriandrum sativum*), squash (*Cucurbita maxima*; Spencer and Snow,  
30 2001, Heredity 86(Pt 6):694-702), linseed/flax (*Linum usitatissimum*; Lamblin et al., 2001,

- Physiol Plant 112:223-232), Brazil nut (*Bertholletia excelsa*), jojoba (*Simmondsia chinensis*), maize (*Zea mays*; Hood et al., 1999, Adv. Exp. Med. Biol. 464:127-147; Hood et al., 1997, Mol. Breed. 3:291-306; Petolino et al., 2000, Transgenic Research 9:1-9), alfalfa (Khouidi et al., 1999, Biotechnol. Bioeng. 64:135-143), tobacco (*Nicotiana tabacum*; Wright et al.,
- 5 Transgenic Res. 10:177-181; Frigerio et al., 2000, Plant Physiol. 123:1483-1493; Cramer et al., 1996, Ann. New York Acad. Sci. 792:62-8-71; Cabanes-Macheteau et al., 1999, Glycobiology 9:365-372; Ruggiero et al., 2000, FEBS Lett. 469:132-136), canola (Bai et al., 2001, Biotechnol. Prog. 17:168-174; Zhang et al., 2000, J. Anim. Sci. 78:2868-2878)), potato (Tacket et al., 1998, J. Infect. Dis. 182:302-305; Richter et al., 2000, Nat. Biotechnol.
- 10 18:1167-1171; Chong et al., 2000, Transgenic Res. 9:71-78), alfalfa (Wigdorovitz et al., 1999, Virology 255:347-353), Pea (*Pisum sativum*; Perrin et al., 2000, Mol. Breed. 6:345-352), rice (*Oryza sativa* ; Stoger et al., 2000, Plant Mol. Biol. 42:583-590), cotton (*Gossypium hirsutum*; Korniyev et al., 2001, Physiol Plant 113:323-331), barley (*Hordeum vulgare*; Petersen et al., 2002, Plant Mol Biol 49:45-58); wheat (*Triticum* spp.; Pellegrineschi et al., 2002, Genome 45:421-430) and bean (*Vicia* spp.; Saalbach et al., 1994, Mol Gen Genet 242:226-236).

If expression of the recombinant nucleic acid is desired in a whole plant rather than in cultured cells, plant cells are first transformed with DNA encoding the peptide, following which, the plant is regenerated. This involves tissue culture procedures that are typically

20 optimized for each plant species. Protocols to regenerate plants are already well known in the art for many species. Furthermore, protocols for other species can be developed by one of skill in the art using routine experimentation. Numerous laboratory manuals are available that describe procedures for plant regeneration, including but not limited to, Smith (2000, Plant tissue culture : techniques and experiments, Academic Press, San Diego), Bhojwani and

25 Razdan (1996, Plant tissue culture : theory and practice, Elsevier Science Pub., Amsterdam), Islam (1996, Plant tissue culture, Oxford & IBH Pub. Co., New Delhi, India), Dodds and Roberts ( 1995, Experiments in plant tissue culture, New York : Cambridge University Press, Cambridge England), Bhojwani (Plant tissue culture : applications and limitations, Elsevier, Amsterdam, 1990), Trigiano and Gray (2000, Plant tissue culture concepts and laboratory

30 exercises,. CRC Press, Boca Raton, Fla), and Lindsey (1991, Plant tissue culture manual : fundamentals and applications, Kluwer Academic, Boston).

While purifying recombinant peptides from plants may potentially be costly, several systems have been developed to minimize these costs. One method directs the synthesized peptide to the seed endosperm from where it can easily be extracted (Wright et al., 2001, Transgenic Res. 10:177-181, Guda et al., 2000, Plant Cell Res. 19:257-262; and U.S. Patent No. 5,767,379, which is incorporated by reference herein in its entirety). An alternative approach is the co-extraction of the recombinant peptide with conventional plant products such as starch, meal or oil. In oil-seed rape, a fusion peptide of oleosin-hurudin when expressed in the plant, attaches to the oil body of the seed, and can be extracted from the plant seed along with the oil (Parmenter, 1995, Plant Mol. Biol. 29:1167-1180; U.S. Patent Nos. 5,650,554, 5,792,922, 5,948,682 and 6,288,304, and US application 2002/0037303, all of which are incorporated in their entirety by reference herein). In a variation on this approach, the oleosin is fused to a peptide having affinity for the exogenous co-expressed peptide of interest (U.S. Patent No. 5,856,452, incorporated by reference herein in its entirety).

Expression of recombinant peptides in plant plastids, such as the chloroplast, generates peptides having no glycan structures attached thereto, similar to the situation in prokaryotes. However, the yield of such peptides is vastly greater when expressed in these plant cell organelles, and thus this type of expression system may have advantages over other systems. For a general review on the technology for plastid expression of exogenous peptides in higher plants, see Hager and Beck (2000, Appl. Microbiol. Biotechnol. 54:302-310, and references cited therein). Plastid expression has been particularly successful in tobacco (see, for example, Staub et al., 2000, Nat. Biotechnol. 18:333-338).

#### F. Transgenic animals

Introduction of a recombinant DNA into the fertilized egg of an animal (e.g., a mammal) may be accomplished using any number of standard techniques in transgenic animal technology. See, e.g., Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986; and U.S. Pat. No. 5,811,634, which is incorporated by reference herein in its entirety. Most commonly, the recombinant DNA is introduced into the embryo by way of pronuclear microinjection (Gordon et al., 1980, PNAS 77:7380-7384; Gordon and Ruddle, 1981, Science 214:1244-1246; Brinster et al., 1981, Cell 27:223-231; Costantini and Lacy, 1981, Nature

294:92-94). Microinjection has the advantage of being applicable to a wide variety of species. Preimplantation embryos may also be transformed with retroviruses (Jaenisch and Mintz, 1974, Proc. Natl. Acad. Sci. U.S.A. 71:1250-1254; Jaenisch et al., 1976, Hamatol Bluttransfus. 19:341-356; Stuhlmann et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:7151-7155). Retroviral mediated transformation has the advantage of adding single copies of the recombinant nucleic acid to the cell, but it produces a high degree of mosaicism. Most recently, embryonic stem cell-mediated techniques have been used (Gossler et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:9065-9069), transfer of entire chromosomal segments (Lavitrano et al., 1989, Cell 57:717-723), and gamete transfection in conjunction with *in vitro* fertilization (Lavitrano et al., 1989, Cell 57:717-723) have also been used. Several books of laboratory procedures have been published disclosing these techniques: Cid-Arregui and García-Carrancá (1998, Microinjection and Transgenesis : Strategies and Protocols, Springer, Berlin), Clarke (2002, Transgenesis Techniques : Principles and Protocols, Humana Press, Totowa, NJ), and Pinkert (1994, Transgenic Animal Technology : A Laboratory Handbook, Academic Press, San Diego).

Once the recombinant DNA is introduced into the egg, the egg is incubated for a short period of time and is then transferred into a pseudopregnant animal of the same species from which the egg was obtained (Hogan et al., supra). In the case of mammals, typically 125 eggs are injected per experiment, approximately two-thirds of which will survive the procedure. Twenty viable eggs are transferred into a pseudopregnant mammal, four to ten of which will develop into live progeny. Typically, 10-30% of the progeny (in the case of mice) carry the recombinant DNA.

While the entire animal can be used as an expression system for the peptides of the invention, in a preferred embodiment, the exogenous peptide accumulates in products of the animal, from which it can be harvested without injury to the animal. In preferred embodiments, the exogenous peptide accumulates in milk, eggs, hair, blood, and urine.

If the recombinant peptide is to be accumulated in the milk of the animal, suitable mammals are ruminants, ungulates, domesticated mammals, and dairy animals. Particularly preferred animals are goats, sheep, camels, cows, pigs, horses, oxen, and llamas. Methods for generating transgenic cows that accumulate a recombinant peptide in their milk are well known: see, Newton (1999, J. Immunol. Methods 231:159-167), Ebert et al. (1991,

Biotechnology 9: 835-838), and U.S. Patent Nos. 6,210,736, 5,849,992, 5,843,705, 5,827,690, 6,222,094, all of which are incorporated herein by reference in their entirety. The generation of transgenic mammals that produce a desired recombinant peptide is commercially available from GTC Biotherapeutics, Framingham, MA.

5        If the recombinant peptide is to be accumulated in eggs, suitable birds include, but are not limited to, chickens, geese, and turkeys. Other animals of interest include, but are not limited to, other species of avians, fish, reptiles and amphibians. The introduction of recombinant DNA to a chicken by retroviral transformation is well known in the art: Thoraval et al. (1995, Transgenic Research 4:369-376), Bosselman et al., (1989, Science 243: 10 533-535), Petropoulos et al. (1992, J. Virol. 66: 3391-3397), U.S. Patent No. 5,162,215, incorporated by reference herein in its entirety. Successful transformation of chickens with recombinant DNA also been achieved wherein DNA is introduced into blastodermal cells and blastodermal cells so transfected are introduced into the embryo: Brazolot et al. (1991, Mol. Reprod. Dev. 30: 304-312), Fraser, et al. (1993, Int. J. Dev. Biol. 37: 381-385), and 15 Petite et al. (1990, Development 108: 185-189). High throughput technology has been developed to assess whether a transgenic chicken expresses the desired peptide (Harvey et al., 2002, Poult. Sci. 81:202-212, U.S. Patent No. 6,423,488, incorporated by reference herein in its entirety). Using retroviral transformation of chicken with a recombinant DNA, exogenous beta-lactamase was accumulated in the egg white of the chicken (Harvey et al., 2002, Nat. 20 Biotechnol. 20(4):396-399). The production of chickens producing exogenous peptides in egg is commercially available from AviGenics, Inc., Athens GA.

#### G. Bacteria

Recombinantly expressed peptides produced in bacteria are not generally glycosylated. However, bacteria systems capable of glycosylating peptides are becoming 25 evident and therefore it is likely that glycosylated recombinant peptides may be produced in bacteria in the future.

Numerous bacterial expression systems are known in the art. Preferred bacterial species include, but are not limited to, *E.coli*. and *Bacillus* species.

The expression of recombinant peptides in *E. coli* is well known in the art. Protocols for *E.* 30 *coli*-based expression systems are found in U.S. Appln No. 20020064835, U.S. Patent Nos. 6,245,539, 5,606,031, 5,420,027, 5,151,511, and RE33,653, among others. Methods to

transform bacteria include, but are not limited to, calcium chloride (Cohen et al., 1972, Proc. Natl. Acad. Sci. U.S.A.. 69:2110-2114; Hanahan, 1983, J. Mol. Biol. 166:557-580; Mandel and Higa, 1970, J. Mol. Biol. 53:159-162) and electroporation (Shigekawa and Dower, 1988, Biotechniques 6:742-751), and those described in Sambrook et al., 2001 (supra). For a  
5 review of laboratory protocols on microbial transformation and expression systems, see Saunders and Saunders (1987, Microbial Genetics Applied to Biotechnology : Principles and Techniques of Gene Transfer and Manipulation, Croom Helm, London), Pühler (1993, Genetic Engineering of Microorganisms, Weinheim, New York), Lee et al., (1999, Metabolic Engineering, Marcel Dekker, New York), Adolph (1996, Microbial Genome Methods, CRC  
10 Press, Boca Raton), and Birren and Lai (1996, Nonmammalian Genomic Analysis : A Practical Guide, Academic Press, San Diego),

For a general review on the literature for peptide expression in *E. coli* see Balbas (2001, Mol. Biotechnol. 19:251-267). Several companies now offer bacterial strains selected for the expression of mammalian peptides, such as the Rosetta™ strains of *E. coli* (Novagen,  
15 inc., Madison, WI; with enhanced expression of eukaryotic codons not normally used in bacteria cells, and enhanced disulfide bond formation),

#### H. Cell engineering

It will be apparent from the present disclosure that the more uniform the starting material produced by a cell, the more efficient will be the generation *in vitro* of large  
20 quantities of peptides having desired glycosylation. Thus, the genetic engineering of host cells to produce uniformly glycosylated peptides as starting material for the *in vitro* enzymatic reactions disclosed herein, provides a significant advantage over using a peptide starting material having a heterogeneous set of glycan structures attached thereto. One preferred peptide starting material for use in the present invention is a peptide having  
25 primarily glycan molecules which consist solely of an elemental trimannosyl core structure. Another preferred starting material is Man3GlcNAc4. Following the remodeling process, the preferred peptides will give rise to the greatest amount of peptides having desired glycosylation, and thus improved clinical efficacy. However, other glycan starting material is also suitable for use in the methods described herein, in that for example, high mannose  
30 glycans may be easily reduced, *in vitro*, to elemental trimannosyl core structures using a series of mannosidases. As described elsewhere herein, other glycan starting material may

also be used, provided it is possible to cleave off all extraneous sugar moieties so that the elemental trimannosyl core structure or Man3GlcNAc4 is generated. Thus, the purpose of using genetically engineered cells for the production of the peptides of the present invention is to generate peptides having as uniform as possible a glycan structure attached thereto, wherein the glycan structure can be remodeled *in vitro* to generate a peptide having desired glycosylation. This will result in a dramatic reduction in production costs of these peptides. Since the glycopeptides produced using this methodology will predominantly have the same N-linked glycan structure, the post-production modification protocol can be standardized and optimized to produce a greater batch-to-batch consistency of final product. As a result, the final completed-chain products may be less heterogeneous than those presently available. The products will have an improved biological half-life and bioactivity as compared to the products of the prior art. Alternatively, if desired, the invention can be used to introduce limited and specific heterogeneity, e.g., by choosing reaction conditions that result in differential addition of sugar moieties.

Preferably, though not as a rigid requirement, the genetically engineered cell is one which produces peptides having glycan structures comprised primarily of an elemental trimannosyl core structure or Man3GlcNAc4. At a minimum, the proportion of these preferred structures produced by the genetically engineered cell must be enough to yield a peptide having desired glycosylation following the remodeling protocol.

In general, any eukaryotic cell type can be modified to become a host cell of the present invention. First, the glycosylation pattern of both endogenous and recombinant glycopeptides produced by the organism are determined in order to identify suitable additions/deletions of enzymatic activities that result in the production of elemental trimannosyl core glycopeptides or Man3GlcNAc4 glycopeptides. This will typically entail deleting activities that use trimannosyl glycopeptides as substrates for a glycosyltransferase reaction and inserting enzymatic activities that degrade more complex N-linked glycans to produce shorter chains. In addition, genetically engineered cells may produce high mannose glycans, which may be cleaved by mannosidase to produce desired starting glycan structures. The mannosidase may be active *in vivo* in the cell (i.e., the cell may be genetically engineered to produce them), or they may be used in *in vitro* post production reactions.

Techniques for genetically modifying host cells to alter the glycosylation profile of expressed peptides are well-known. See, e.g., Altmann et al. (1999, Glycoconjugate J. 16: 109-123), Ailor et al. (2000, Glycobiology 10(8): 837-847), Jarvis et al., (*In vitro* Conference, March, 1999, abstract), Hollister and Jarvis, (2001, Glycobiology 11(1): 1-9),  
5 and Palacpac et al., (1999, PNAS USA 96: 4697), Jarvis et al., (1998, Curr. Opin. Biotechnol. 9:528-533), Gerngross (U.S. Patent Publication No. 20020137134), all of which disclose techniques to "mammalianize" insect or plant cell expression systems by transfecting insect or plant cells with glycosyltransferase genes.

Techniques also exist to genetically alter the glycosylation profile of peptides  
10 expressed in *E. coli*. *E. coli* has been engineered with various glycosyltransferases from the bacteria *Neisseria meningitidis* and *Azorhizobium* to produce oligosaccharides *in vivo* (Bettler et al., 1999, Glycoconj. J. 16:205-212). *E. coli* which has been genetically engineered to over-express *Neisseria meningitidis*  $\beta$ 1,3 N acetyl glucoaminytransferase lgtA gene will efficiently glycosylate exogenous lactose (Priem et al., 2002, Glycobiology 12:235-240).

15 Fungal cells have also been genetically modified to produce exogenous glycosyltransferases (Yoshida et al., 1999, Glycobiology, 9(1):53-58; Kalsner et al., 1995, Glycoconj. J. 12:360-370; Schwientek and Ernst, 1994, Gene 145(2):299-303; Chiba et al, 1995, Biochem J. 308:405-409).

Thus, in one aspect, the present invention provides a cell that glycosylates a  
20 glycopeptide population such that a proportion of glycopeptides produced thereby have an elemental trimannosyl core or a Man3GlcNAc4 structure. Preferably, the cell produces a peptide having a glycan structure comprised solely of an elemental trimannosyl core. At a minimum, the proportion of peptides having an elemental trimannosyl core or a Man3GlcNAc4 structure is enough to yield peptides having desired glycosylation following  
25 the remodeling process. The cell has introduced into it one or more heterologous nucleic acid expression units, each of which may comprise one or more nucleic acid sequences encoding one or more peptides of interest. The natural form of the glycopeptide of interest may comprise one or more complex N-linked glycans or may simply be a high mannose glycan.

The cell may be any type of cell and is preferably a eukaryotic cell. The cell may be a  
30 mammalian cell such as human, mouse, rat, rabbit, hamster or other type of mammalian cell. When the cell is a mammalian cell, the mammalian cell may be derived from or contained



within a non-human transgenic mammal where the cell in the mammal encodes the desired glycopeptide and a variety of glycosylating and glycosidase enzymes as necessary for the production of desired glycopeptide molecules. In addition, the cell may be a fungal cell, preferably, a yeast cell, or the cell may be an insect or a plant cell. Similarly, when the cell is a plant cell, the plant cell may be derived from or contained within a transgenic plant, wherein the plant encodes the desired glycopeptide and a variety of glycosylating and glycosidase enzymes as are necessary for the production of desired glycopeptide molecules.

In some embodiments the host cell may be a eukaryotic cell expressing one or more heterologous glycosyltransferase enzymes and/or one or more heterologous glycosidase enzymes, wherein expression of a recombinant glycopeptide in the host cell results in the production of a recombinant glycopeptide having an elemental trimannosyl core as the primary glycan structure attached thereto.

In some embodiments the heterologous glycosyltransferase enzyme useful in the cell may be selected from a group consisting of any known glycosyltransferase enzyme included for example, in the list of Glycosyltransferase Families available in Taniguchi et al. (2002, Handbook of Glycosyltransferases and Related Genes, Springer, New York).

In other embodiments, the heterologous glycosylase enzyme may be selected from a group consisting of mannosidase 1, mannosidase 2, mannosidase 3, and other mannosidases, including, but not limited to, microbial mannosidases. Additional disclosure regarding enzymes useful in the present invention is provided elsewhere herein.

In yet other embodiments, the host cell may be a eukaryotic cell wherein one or more endogenous glycosyltransferase enzymes and/or one or more endogenous glycosidase enzymes have been inactivated such that expression of a recombinant glycopeptide in the host cell results in the production of a recombinant glycopeptide having an elemental trimannosyl core as the primary glycan structure attached thereto.

In additional embodiments, the host cell may express heterologous glycosyltransferase enzymes and/or glycosidase enzymes while at the same time one or more endogenous glycosyltransferase enzymes and/or glycosidase enzymes are inactivated. Endogenous glycosyltransferase enzymes and/or glycosidase enzymes may be inactivated using any technique known to those skilled in the art including, but not limited to, antisense techniques and techniques involving insertion of nucleic acids into the genome of the host

cell. In some embodiments, the endogenous enzymes may be selected from a group consisting of GnT-I, a selection of mannosidases, xylosyltransferase, core  $\alpha$ 1,3 fucosyltransferase, serine/threonine O-mannosyltransferases, and the like.

Alternatively, an expression system that naturally glycosylates peptides such that the N-linked glycans are predominantly the trimannosyl core type, or the Man3GlcNAc4 type, can be exploited. An example of a cell type that produces the trimannosyl core is Sf9 cells. Other such expression systems can be identified by analyzing glycopeptides that are naturally or recombinantly expressed in cells and selecting those which exhibit the desired glycosylation characteristics. The invention should be construed to include any and all such cells for the production of the peptides of the present invention.

#### V. Purification of glycan remodeled and/or glycoconjugated peptides

If the modified glycoprotein is produced intracellularly or secreted, as a first step, the particulate debris, either host cells, lysed fragments, is removed, for example, by centrifugation or ultrafiltration; optionally, the protein may be concentrated with a commercially available protein concentration filter, followed by separating the peptide variant from other impurities by one or more steps selected from immunoaffinity chromatography, ion-exchange column fractionation (*e.g.*, on diethylaminoethyl (DEAE) or matrices containing carboxymethyl or sulfopropyl groups), chromatography on Blue-Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toyopearl, Butyl Toyopearl, Phenyl Toyopearl, or protein A Sepharose, SDS-PAGE chromatography, silica chromatography, chromatofocusing, reverse phase HPLC (RP-HPLC), gel filtration using, *e.g.*, Sephadex molecular sieve or size-exclusion chromatography, chromatography on columns that selectively bind the peptide, and ethanol, pH or ammonium sulfate precipitation, membrane filtration and various techniques.

Modified peptides produced in culture are usually isolated by initial extraction from cells, enzymes, etc., followed by one or more concentration, salting-out, aqueous ion-exchange, or size-exclusion chromatography steps. Additionally, the modified glycoprotein may be purified by affinity chromatography. Then, HPLC may be employed for final purification steps.

A protease inhibitor, *e.g.*, phenylmethylsulfonylfluoride (PMSF) may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

Within another embodiment, supernatants from systems which produce the modified peptide of the invention are first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate may be applied to a suitable purification matrix. For example, a suitable affinity matrix may comprise a ligand for the peptide, a lectin or antibody molecule bound to a suitable support. Alternatively, an anion-exchange resin may be employed, for example, a matrix or substrate having pendant DEAE groups. Suitable matrices include acrylamide, agarose, dextran, cellulose, or other types commonly employed in protein purification. Alternatively, a cation-exchange step may be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are particularly preferred.

Then, one or more RP-HPLC steps employing hydrophobic RP-HPLC media, *e.g.*, silica gel having pendant methyl or other aliphatic groups, may be employed to further purify a peptide variant composition. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous modified glycoprotein.

The modified peptide of the invention resulting from a large-scale fermentation may be purified by methods analogous to those disclosed by Urdal *et al.*, *J. Chromatog.* 296: 171 (1984). This reference describes two sequential, RP-HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column. Alternatively, techniques such as affinity chromatography may be utilized to purify the modified glycoprotein.

#### VI. Preferred Peptides and Nucleic Acids Encoding Preferred Peptides

The present invention includes isolated nucleic acids encoding various peptides and proteins, and similar molecules or fragments thereof. Such peptides include, but are not limited to human granulocyte colony stimulating factor (G-CSF), human interferon alpha (IFN-alpha), human interferon beta (IFN-beta), human Factor VII (Factor VII), human Factor IX (Factor IX), human follicle stimulating hormone (FSH), human erythropoietin (EPO), human granulocyte/macrophage colony stimulating factor (GM-CSF), human interferon

gamma (IFN-gamma), human alpha-1-protease inhibitor (also known as alpha-1-antitrypsin or alpha-1-trypsin inhibitor; A-1-PI), glucocerebrosidase, human tissue-type activator (TPA), human interleukin-2 (IL-2), human Factor VIII (Factor VIII), a 75 kDa tumor necrosis factor receptor fused to a human IgG immunoglobulin Fc portion, commercially known as

5 ENBREL™ or ETANERCEPT™ (chimeric TNFR), human urokinase (urokinase), a Fab fragment of the human/mouse chimeric monoclonal antibody that specifically binds glycoprotein IIb/ IIIa and the vitronectin alpha<sub>v</sub> beta<sub>3</sub> receptor, known commercially as REOPRO™ or ABCIXIMAB (chimeric anti-glycoprotein IIb/IIIa), a mouse/human chimeric monoclonal antibody that specifically binds human HER2, known commercially as

10 HERCEPTIN™ (chimeric anti-HER2), a human/mouse chimeric antibody that specifically binds the A antigenic site or the F protein of respiratory syncytial virus commercially known as SYNAGIST™ or PALIVIZUMAB (chimeric anti-RSV), a chimeric human/mouse monoclonal antibody that specifically binds CD20 on human B-cells, known commercially as RITUXAN™ or RITUXAMAB (chimeric anti-CD20), human recombinant DNase (DNase),

15 a chimeric human/mouse monoclonal antibody that specifically binds human tumor necrosis factor, known commercially as REMICADE™ or INFLIXIMAB (chimeric anti-TNF), human insulin, the surface antigen of a hepatitis B virus (adw subtype; HBsAg), and human growth hormone (HGH), and the like.

The isolated nucleic acid of the invention should be construed to include an RNA or a

20 DNA sequence encoding any of the above-identified peptides of the invention, and any modified forms thereof, including chemical modifications of the DNA or RNA which render the nucleotide sequence more stable when it is cell free or when it is associated with a cell. As a non-limiting example, oligonucleotides which contain at least one phosphorothioate modification are known to confer upon the oligonucleotide enhanced resistance to nucleases.

25 Specific examples of modified oligonucleotides include those which contain phosphorothioate, phosphotriester, methyl phosphonate, short chain alkyl or cycloalkyl intersugar linkages, or short chain heteroatomic or heterocyclic intersugar ("backbone") linkages. In addition, oligonucleotides having morpholino backbone structures (U.S. Patent No: 5,034,506) or polyamide backbone structures (Nielsen et al., 1991, Science 254: 1497)

30 may also be used.

Chemical modifications of nucleotides may also be used to enhance the efficiency with which a nucleotide sequence is taken up by a cell or the efficiency with which it is expressed in a cell. Any and all combinations of modifications of the nucleotide sequences are contemplated in the present invention.

5       The present invention should not be construed as being limited solely to the nucleic and amino acid sequences disclosed herein. As described in more detail elsewhere herein, once armed with the present invention, it is readily apparent to one skilled in the art that other nucleic acids encoding the peptides of the present invention can be obtained by following the procedures described herein (*e.g.*, site-directed mutagenesis, frame shift mutations, and the  
10   like), and procedures that are well-known in the art.

Also included are isolated nucleic acids encoding fragments of peptides, wherein the peptide fragments retain the desired biological activity of the peptide. In addition, although exemplary nucleic acids encoding preferred peptides are disclosed herein in relation to specific SEQ ID NOS, the invention should in no way be construed to be limited to any  
15   specific nucleic acid disclosed herein. Rather, the invention should be construed to include any and all nucleic acid molecules having a sufficient percent identity with the sequences disclosed herein such that these nucleic acids also encode a peptide having the desired biological activity disclosed herein. Also contemplated are isolated nucleic acids that are shorter than full length nucleic acids, wherein the biological activity of the peptide encoded  
20   thereby is retained. Methods to determine the percent identity between one nucleic acid and another are disclosed elsewhere herein as are assays for the determination of the biological activity of any specific preferred peptide.

Also as disclosed elsewhere herein, any other number of procedures may be used for the generation of derivative, mutant, or variant forms of the peptides of the present invention  
25   using recombinant DNA methodology well known in the art such as, for example, that described in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York) and Ausubel et al. (1997, Current Protocols in Molecular Biology, Green & Wiley, New York). Procedures for the introduction of amino acid changes in a peptide or polypeptide by altering the DNA sequence encoding the peptide  
30   are well known in the art and are also described in Sambrook et al. (1989, *supra*); Ausubel et al. (1997, *supra*).

The invention includes a nucleic acid encoding a G-CSF, IFN-alpha, IFN-beta, Factor VII, Factor IX, FSH, EPO, GM-CSF, IFN-gamma, A-1-PI, glucocerebrosidase, TPA, IL-2, Factor VIII, chimeric TNFR, urokinase, chimeric anti-glycoprotein IIb/IIa, chimeric anti-HER2, chimeric anti-RSV, chimeric anti-CD20, DNase, chimeric anti-TNF, human insulin, HBsAg, and HGH, wherein a nucleic acid encoding a tag peptide is covalently linked thereto. That is, the invention encompasses a chimeric nucleic acid wherein the nucleic acid sequence encoding a tag peptide is covalently linked to the nucleic acid encoding a peptide of the present invention. Such tag peptides are well known in the art and include, for instance, green fluorescent protein (GFP), myc, myc-pyruvate kinase (myc-PK), His<sub>6</sub>, maltose binding protein (MBP), an influenza virus hemagglutinin tag polypeptide, a flag tag polypeptide (FLAG), and a glutathione-S-transferase (GST) tag polypeptide. However, the invention should in no way be construed to be limited to the nucleic acids encoding the above-listed tag peptides. Rather, any nucleic acid sequence encoding a peptide which may function in a manner substantially similar to these tag peptides should be construed to be included in the present invention.

The nucleic acid comprising a nucleic acid encoding a tag peptide can be used to localize a peptide of the present invention within a cell, a tissue, and/or a whole organism (e.g., a mammalian embryo), detect a peptide of the present invention secreted from a cell, and to study the role(s) of the peptide in a cell. Further, addition of a tag peptide facilitates isolation and purification of the "tagged" peptide such that the peptides of the invention can be produced and purified readily.

The invention includes the following preferred isolated peptides: G-CSF, IFN-alpha, IFN-beta, Factor VII, Factor IX, FSH, EPO, GM-CSF, IFN-gamma, A-1-PI, glucocerebrosidase, TPA, IL-2, Factor VIII, chimeric TNFR, urokinase, chimeric anti-glycoprotein IIb/IIIa, chimeric anti-HER2, chimeric anti-RSV, chimeric anti-CD20, DNase, chimeric anti-TNF, human insulin, HBsAg, and HGH.

The present invention should also be construed to encompass "derivatives," "mutants", and "variants" of the peptides of the invention (or of the DNA encoding the same) which derivatives, mutants, and variants are peptides which are altered in one or more amino acids (or, when referring to the nucleotide sequence encoding the same, are altered in one or more base pairs) such that the resulting peptide (or DNA) is not identical to the sequences

recited herein, but has the same biological property as the peptides disclosed herein, in that the peptide has biological/biochemical properties of G-CSF, IFN-alpha, IFN-beta, Factor VII, Factor IX, FSH, EPO, GM-CSF, IFN-gamma, A-1-PI, glucocerebrosidase, TPA, IL-2, Factor VIII, chimeric TNFR, urokinase, chimeric anti-glycoprotein IIb/IIIa, chimeric anti-HER2, 5 chimeric anti-RSV, chimeric anti-CD20, DNase, chimeric anti-TNF, human insulin, HBsAg, and HGH.

Further included are fragments of peptides that retain the desired biological activity of the peptide irrespective of the length of the peptide. It is well within the skill of the artisan to isolate smaller than full length forms of any of the peptides useful in the invention, and to 10 determine, using the assays provided herein, which isolated fragments retain a desired biological activity and are therefore useful peptides in the invention.

A biological property of a protein of the present invention should be construed to include, but not be limited to include the ability of the peptide to function in the biological assay and environments described herein, such as reduction of inflammation, elicitation of an 15 immune response, blood-clotting, increased hematopoietic output, protease inhibition, immune system modulation, binding an antigen, growth, alleviation of treatment of a disease, DNA cleavage, and the like.

#### A. G-CSF

20 The present invention encompasses a method for the modification of the glycan structure on G-CSF. G-CSF is well known in the art as a cytokine produced by activated T-cells, macrophages, endothelial cells, and stromal fibroblasts. G-CSF primarily acts on the bone marrow to increase the production of inflammatory leukocytes, and further functions as an endocrine hormone to initiate the replenishment of neutrophils consumed during 25 inflammatory functions. G-CSF also has clinical applications in bone marrow replacement following chemotherapy.

While G-CSF has been shown to be an important and useful compound for therapeutic applications in mammals, especially humans, present methods for the production of G-CSF from recombinant cells results in a product having a relatively short biological life, 30 an inaccurate glycosylation pattern that could potentially lead to immunogenicity, loss of

function, and an increased need for both larger and more frequent doses in order to achieve the same effect, and the like.

G-CSF has been isolated and cloned, the nucleic acid and amino acid sequences of which are presented as SEQ ID NO:1 and SEQ ID NO:2, respectively (Figure 52A and 52B, respectively). The present invention encompasses a method for modifying G-CSF, particularly as it relates to the ability of G-CSF to function as a potent and functional biological molecule. The skilled artisan, when equipped with the present disclosure and the teachings herein, will readily understand that the present invention provides compositions and methods for the modification of G-CSF.

The present invention further encompasses G-CSF variants, as well known in the art. As an example, but in no way meant to be limiting to the present invention, a G-CSF variant has been described in U.S. Patent No. 6,166,183, in which a G-CSF comprising the natural complement of lysine residues and further bound to one or two polyethylene glycol molecules is described. Additionally, U.S. Patent Nos. 6,004,548, 5,580,755, 5,582,823, and 5,676,941 describe a G-CSF variant in which one or more of the cysteine residues at position 17, 36, 42, 64, and 74 are replaced by alanine or alternatively serine. U.S. Patent No. 5,416,195 describes a G-CSF molecule in which the cysteine at position 17, the aspartic acid at position 27, and the serines at positions 65 and 66 are substituted with serine, serine, proline, and proline, respectively. Other variants are well known in the art, and are described in, for example, U.S. Patent No. 5,399,345.

The expression and activity of a modified G-CSF molecule of the present invention can be assayed using methods well known in the art, and as described in, for example, U.S. Patent No. 4,810,643. As an example, activity can be measured using radio-labeled thymidine uptake assays. Briefly, human bone marrow from healthy donors is subjected to a density cut with Ficoll-Hypaque (1.077 g/ml, Pharmacia, Piscataway, NJ) and low density cells are suspended in Iscove's medium (GIBCO, La Jolla, CA) containing 10% fetal bovine serum, glutamine and antibiotics. About  $2 \times 10^4$  human bone marrow cells are incubated with either control medium or the G-CSF or the present invention in 96-well flat bottom plates at about 37° C in 5% CO<sub>2</sub> in air for about 2 days. Cultures are then pulsed for about 4 hours with 0.5 µCi/well of <sup>3</sup>H-thymidine (New England Nuclear, Boston, Mass.) and uptake is measured as described in, for example, Ventua, et al.(1983, Blood 61:781). An increase in



<sup>3</sup>H-thymidine incorporation into human bone marrow cells as compared to bone marrow cells treated with a control compound is an indication of a active and viable G-CSF compound.

#### B. IFN alpha and IFN beta

5       The present invention further encompasses a method for the remodeling and modification of IFN alpha and IFN beta. IFN alpha is part of a family of approximately twenty peptides of approximately 18kDa in weight. IFN alpha and IFN beta, collectively known as the Type I interferons, bind to the same cellular receptor and elicit similar responses. Type I IFNs inhibit viral replication, increase the lytic potential of NK cells,  
10       modulate MHC molecule expression, and inhibit cellular proliferation, among other things. Type I IFN has been used as a therapy for viral infections, particularly hepatitis viruses, and as a therapy for multiple sclerosis.

      Current compositions of Type I IFN are, as described above, useful compounds for both the modulation of aberrant immunological responses and as a therapy for a variety of  
15       diseases. However, they are hampered by decreased potency and function, and a limited half-life in the body as compared to natural cytokines comprising the natural complement of glycosylation.

      The prototype nucleotide and amino acid sequence for IFN alpha is set forth herein as SEQ ID NO:3 and SEQ ID NO:4, respectively (Figure 53A and 53B, respectively). IFN beta  
20       comprises a single gene product of approximately 20 kDa, the nucleic acid and amino acid sequence of which are presented herein as SEQ ID NO:5 and SEQ ID NO:6 (Figure 54A and 54B, respectively). The present invention is not limited to the nucleotide and amino acid sequences herein. One of skill in the art will readily appreciate that many variants of IFN alpha exist both naturally and as engineered derivatives. Similarly, IFN beta has been  
25       modified in attempts to achieve a more beneficial therapeutic profile. Examples of modified Type I IFNs are well known in the art (see Table 8), and are described in, for example U.S. Patent No. 6,323,006, in which cysteine-60 is substituted for tyrosine, U. S. Patent Nos. 4,737,462, 4,588,585, 5,545,723, and 6,127,332 where an IFN beta with a substitution of a variety of amino acids is described. Additionally, U.S. Patent Nos. 4,966,843, 5,376,567,  
30       5,795,779 describe IFN alpha-61 and IFN-alpha-76. U.S. Patent Nos. 4,748,233 and 4,695,543 describe IFN alpha gx-1, whereas U.S. Patent No. 4,975,276 describes IFN alpha-

54. In addition, U.S. Patent Nos. 4,695,623, 4,897,471, 5,661,009, and 5,541,293 all describe a consensus IFN alpha sequence to represent all variants known at the date of filing. While this list of Type I IFNs and variants thereof is in no way meant to be exhaustive, one of skill in the art will readily understand that the present invention encompasses IFN beta and IFN alpha molecules, derivatives, and variants known or to be discovered in the future.

Table 8. Interferon- $\alpha$  Isoforms.

$\alpha$ type	AA characteristic
1a	A <sup>114</sup>
1b	V <sup>114</sup>
2a	K <sup>23</sup> -H <sup>34</sup>
2b	R <sup>23</sup> -H <sup>34</sup>
2c	R <sup>23</sup> -R <sup>34</sup>
4a	A <sup>51</sup> -E <sup>114</sup>
4b	T <sup>51</sup> -V <sup>114</sup>
7a	M <sup>132</sup> -K <sup>159</sup> -G <sup>161</sup>
7b	M <sup>132</sup> -Q <sup>159</sup> -R <sup>161</sup>
7c	T <sup>132</sup> -K <sup>159</sup> -G <sup>161</sup>
8a	V <sup>98</sup> -L <sup>99</sup> -C <sup>100</sup> -D <sup>101</sup> -R <sup>161</sup>
8b	S <sup>98</sup> -C <sup>99</sup> -V <sup>100</sup> -M <sup>101</sup> -R <sup>161</sup>
8c	S <sup>98</sup> -C <sup>99</sup> -V <sup>100</sup> -M <sup>101</sup> -D <sup>161</sup> $\Delta$ (162-166)
10a	S <sup>8</sup> -L <sup>89</sup>
10b	T <sup>8</sup> -I <sup>89</sup>
14a	F <sup>152</sup> -Q <sup>159</sup> -R <sup>161</sup>
14b	F <sup>152</sup> -K <sup>159</sup> -G <sup>161</sup>
14c	L <sup>152</sup> -Q <sup>159</sup> -R <sup>161</sup>
17a	P <sup>34</sup> -S <sup>55</sup> -I <sup>161</sup>
17b	H <sup>34</sup> -S <sup>55</sup> -I <sup>161</sup>
17c	H <sup>34</sup> -S <sup>55</sup> -R <sup>161</sup>
17d	H <sup>34</sup> -P <sup>55</sup> -R <sup>161</sup>
21a	M <sup>96</sup>
21b	L <sup>96</sup>

10 Methods of expressing IFN in recombinant cells are well known in the art, and is easily accomplished using techniques described in, for example U.S. Patent No. 4,966,843, and in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York) and Ausubel et al. (1997, Current Protocols in Molecular Biology, Green & Wiley, New York).